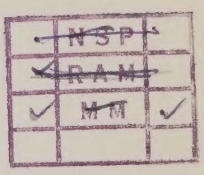
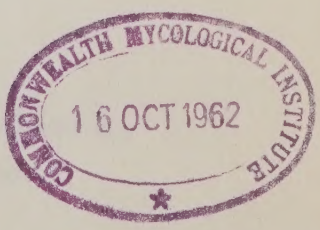
# ANTIMICROBIAL AGENTS AND CHEMOTHERAPY - 1961

[ Successor to Antimicrobial Agents Annual ]



# ANTIMICROBIAL AGENTS AND CHEMOTHERAPY — 1961

Proceedings of the First Interscience Conference on Antimicrobial Agents and Chemotherapy, New York City, October 31 – November 2, 1961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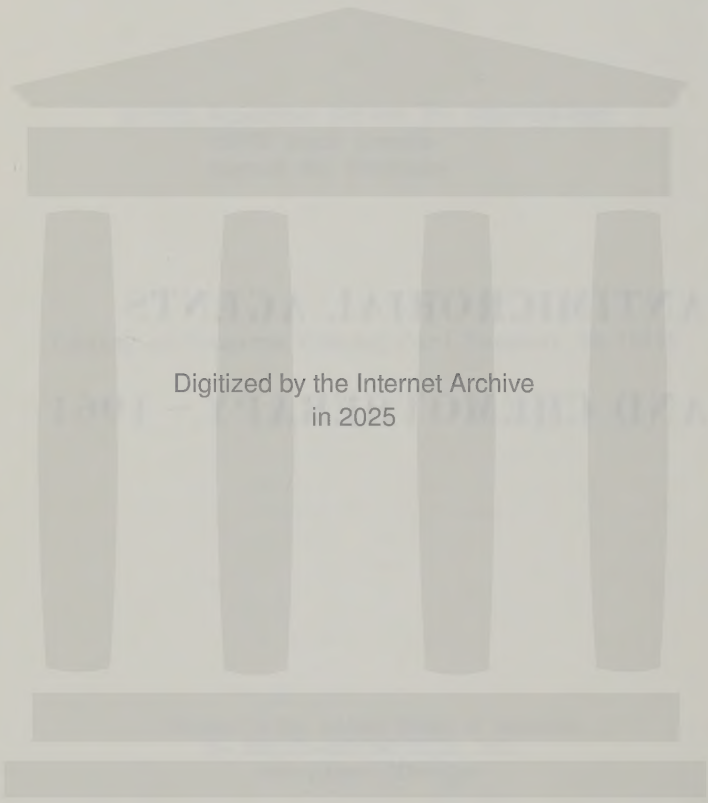
# ANTIMICROBIAL AGENTS

# AND CHEMOTHERAPY – 1961



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# Preface

In any field of scientific endeavor a medium for the mutual exchange of ideas and information among the investigators is both desirable and essential. This is particularly true when the subject of investigation overlaps several related disciplines. In extension of its aim to advance scientific knowledge in microbiology and allied fields, the American Society for Microbiology has embarked upon a program of interscience conferences which are designed to provide the opportunity for intercommunication between microbiologists and investigators in related disciplines. To this end, a Committee on Interscience Conferences, under the chairmanship of Dr. Orville Wyss, has been established and charged with the encouragement and general supervision of conferences of an interdisciplinary nature, independent of the present framework of the Society's Annual Meeting.

The broad area of antimicrobial agents and chemotherapy covers a wide spectrum of research activity in the fields of microbiology, chemistry, pharmacology, biochemistry, clinical medicine, and pathology. Although these disciplines are diverse, they nevertheless are characterized by numerous and varied interrelationships which provide bonds of common interest. In late 1960, in response to a petition by a number of investigators whose interests lie in those several disciplines, the Council Policy Committee of the Society authorized the sponsorship of an Interscience Conference on Antimicrobial Agents and Chemotherapy and assumed the responsibility for publication of the proceedings of the Conference. It is planned that this Conference will be held annually. To insure its permanence on a continuing basis, appropriate mechanisms were set up by the Council Policy Committee.

The first of such conferences was held October 31 to November 2, 1961, the proceedings of which are presented in this *Annual*. The obvious success of this meeting raises the hope that subsequent Interscience Conferences of this or a comparable nature will furnish tangible evidence of the desire of the Society to be of service to microbiologists and their colleagues in related scientific fields.

The Society is deeply indebted to all of the individuals who contributed of their time and effort—through membership on the several committees or through personal service at the time of the meetings—to the effective organization and operation of the Conference. They were ably assisted by the Executive Secretary and the Managing Editor of the Society's headquarters staff. A special word of thanks is due to the Executive Committee of the Conference, which was composed of Dr. George M. Savage (Chairman), Dr. Maxwell Finland, and Dr. John C. Sylvester.

JOHN E. BLAIR, *President*

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# NOSOCOMIAL INFECTIONS WITH *HERELLEA VAGINICOLA* IN BURNED PATIENTS

CHARLES D. GRABER, ERWIN R. RABIN,
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The term "therapy-potentiated infection" is applied to hospital patients in whom intense, protracted antibiotic treatment has resulted in invasion by an organism which normally would not cause disease. It is to be regarded as the price exacted for a necessary and frequently life-saving procedure. These infections are most often caused by commensal organisms which behave as opportunistic invaders. Thus, intestinal candidiasis may follow prolonged oral antibiotic administration.

An alarming development of the past decade of antibiotic therapy has been the appearance of saprophytes which behave as pathogens, probably because of a disturbance of the equilibrium of the microbial flora by antibiotics. Thus, *Bacillus subtilis* (Cox, Sockwell, and Landers, 1959) and *Serratia marcescens* (Graber et al., 1960) have been reported as agents of septicemia in man. The most important member of this group of commensal bacteria is undoubtedly *Pseudomonas aeruginosa,* strains of which have shown a remarkable potentiality for invading severely debilitated patients during prolonged antibiotic therapy. This is especially noteworthy in the seriously burned patient (Rabin, Graber, and Vogel, *in press;* Tumbusch et al., 1961). No antibiotics have yet been shown to cope with such infections.

Bacterial invasion of patients in the burn ward offers an excellent environment for studying therapy-potentiated infection, since the initial open wound, followed by an exuding eschar, calls for intensive antibiotic prophylaxis to avert bacteremia. Under these conditions, our unit has noted the appearance, with increasing frequency, of infections with an organism of obscure taxonomic position, corresponding to the description of *Herellea vaginicola* as given by De Bord (1942). There have probably been other binomials applied to this organism, including the *B. anitratum* of Schaub and Hauber (1948). The *Diplococcus mucosum* of Von Lingelsheim (1905) may have been the same organism. Many European taxonomists appear to favor the classification of *Moraxella lwoffi* (Andreau, 1940). The organisms are gram-negative to gram-variable cocco-bacilli, pleomorphic,

relatively inert toward carbohydrates, and most are probably to be placed in the family *Parvobacteriaceae*, according to the current consensus of taxonomists. A principal biochemical feature is the inability of these organisms to reduce nitrate to nitrite. The ambiguous taxonomic status of these organisms is such that they were omitted from the 7th edition of Bergey's Manual of Determinative Bacteriology.

This report documents the appearance of *H. vaginicola* as a surface contaminant in burn wounds of patients at this unit, its appearance in bacteremia accompanying such infections, and the antibiotic refractoriness of these organisms.

## Experimental

Notation of a recovery of a culture of *H. vaginicola* at this unit was first made early in 1960, when positive blood cultures were obtained on three successive days from one patient. No *Herellea* organisms had been reported isolated from burned surfaces or blood cultures prior to this time. After the recognition of the first bacteremic strain, a greater awareness of the possible presence of *H. vaginicola* developed. During the remainder of 1960 and the first 6 months of 1961, cultures from 68 of 133 patients were positive, with a total of 362 isolations being made (Table 1).

TABLE 1. *Isolation of Herellea vaginicola from burned patients*

| Year | Surface cultures | | | Blood cultures | |
|---|---|---|---|---|---|
| | Total patients | Patients positive | Total isolates | Antemortem | Postmortem |
| 1960 | 99 | 50 | 260 | 3 | 5* |
| 1961 | 34 | 18 | 102 | 0 | 0 |
| *Total* | 133 | 68 | 362 | 3 | 5 |

*One patient positive antemortem as well as postmortem.

During this period, a total of seven patients yielded positive blood cultures for this organism. Three of these were antemortem, and four postmortem, with one patient positive both ante- and postmortem. Another patient exhibited a positive terminal blood culture, as well as a postmortem positive. The three antemortem patients showed positive cultures on the 4th to 6th, 11th to 16th, and 5th to 8th postburn days, respectively.

All of the patients with positive *Herellea* blood cultures died, but except for one patient, the *H. vaginicola* from the blood stream always accompanied either *P. aeruginosa* or *P. aeruginosa* with yet a third organism. The five postmortem positive blood cultures were usually accompanied by *Pseudomonas*. In two patients with positive

*Herellea* blood cultures during their illness, administration of colistin sulfate was followed by disappearance of this organism from the blood stream. One of these patients succumbed after several subsequent blood cultures positive for *Pseudomonas;* the second showed mixed *Staphylococcus aureus* and *P. aeruginosa* in the blood stream at death.

*H. vaginicola* grows well on routine laboratory media, and on blood agar forms large, round, smooth, nonhemolytic, gray colonies. The strains here observed grew well on eosin-methylene blue agar and on MacConkey's agar. The strains fermented glucose, xylose, and arabinose without gas production. Lactose, sucrose, salicin, and mannitol were not fermented in conventional 1% solutions. However, *Herellea* strains exhibited the peculiar ability to ferment concentrated but not dilute lactose, and all strains observed formed acid in 10% semisolid agar. The bacteremic strains were found to agglutinate strongly in antiserum for the Communicable Disease Center (CDC) *H. vaginicola* strain 101. Weak agglutination occurred in antisera for CDC strains 1043, 39, 26, and 969. The strains observed were thus closely related or identical, culturally and serologically, with CDC strain *H. vaginicola* 101.

TABLE 2. *Disc and tube sensitivity of Herellea vaginicola to selected antibiotics**

| Isolates | Pen (10 units) | Chloro (30 μg) | Erythro (15 μg) | Terra (30 μg) | Kanamycin (30 μg) | Neomycin (30 μg) | Poly B (30 μg) | Colistin (30 μg) |
|---|---|---|---|---|---|---|---|---|
| 362 | < 1 | 45 | 79 | 73 | 96 | 98 | 95 | 93 |
| C.P.† | | | | | | | | |
| (2-7-1) | > 25 | > 25 | 6.2 | 3.1 | 6.2 | 6.2 | 0.39 | 0.39 |
| (2-8-2) | > 25 | > 25 | 6.2 | 3.1 | 12.5 | 6.2 | 0.39 | 0.39 |
| M.M.† | | | | | | | | |
| (5-22-5) | > 25 | > 25 | .78 | .78 | 3.1 | 3.1 | 3.1 | 0.78 |
| (5-27-1) | > 25 | > 25 | .78 | .78 | 6.2 | 6.2 | 6.2 | 0.39 |
| C.S.† | | | | | | | | |
| (5-28-3) | > 25 | > 25 | > 25 | – | 3.1 | 3.1 | 3.1 | 1.5 |
| (5-59-1) | > 25 | > 25 | > 25 | > 25 | 3.1 | 6.2 | 12.5 | 1.5 |

*The paper-disc method was used to determine the per cent sensitivity of all isolates (first row). Pen = penicillin; Chloro = chloramphenicol; Erythro = erythromycin; Terra = oxytetracycline (Terramycin); Poly B = polymyxin B.

†These bacteremic strains all showed, in addition to the results listed, insensitivity to more than 25 μg streptomycin, vancomycin, and bacitracin. These strains were tested by the tube dilution method; results are in μg/ml.

When tested by the disc method for antibiotic sensitivity, the 362 *H. vaginicola* strains were in the main relatively resistant to penicillin and to the broad-spectrum antibiotics (Table 2). Less than half of the strains were sensitive to chloramphenicol, but three-fourths of the strains were sensitive to oxytetracycline and almost four-fifths were sensitive to erythromycin. Almost all strains were sensitive to kanamycin, neomycin, polymyxin B, and colistin sulfate.

The sensitivity of strains from the three patients with *Herellea* bacteremia during life were studied by tube dilution method. The strains were resistant to penicillin, chloramphenicol, streptomycin, vancomycin, and bacitracin. All were relatively sensitive to neomycin, kanamycin, polymyxin B, and colistin sulfate. Marked variations were noted in sensitivity to erythromycin and to oxytetracycline.

## Discussion

Although there have been increasingly frequent reports (Schaub and Hauber, 1948; Weed, 1961) of organisms of the tribe *Mimae* being observed as etiological agents of disease, they are evidently unfamiliar to a relatively large proportion of bacteriologists. The lack of a generally accepted taxonomic position for this group of organisms may account for their omission from the current Bergey's Manual. The belated recognition of *Herellea* in our laboratory emphasizes the need for wider knowledge of these organisms. An appeal for reports of infections due to *Mimae* was recently made by Weed (1961) of the Mayo Foundation.

It is virtually certain that *H. vaginicola* was being overlooked in surface cultures from burned patients in our laboratory prior to 1960. When the organism was found in the blood stream, more detailed study of colonies resembling those of *Enterobacteriaceae* revealed the group to be relatively frequent inhabitants of burned surfaces. The appearance of this organism in bacteremia may connote increased invasive capability on the part of some strains, but is more probably evidence of increasing resistance to antibiotics. The strain was streptomycin-insensitive, and the prophylactic regimen of penicillin and streptomycin which almost all burned patients received during the period of observation may well have contributed to permitting the organism selectively to survive. The strain was indeed a definite invader of the blood stream in at least three patients, since multiple isolations were obtained from each individual during life.

## Literature Cited

Andreau, A. 1940. Etude du genre Moraxella. Ann. inst. Pasteur 64:126-166.

Cox, R., G. Sockwell, and B. Landers. 1959. Bacillus subtilis septicemia. N. Engl. J. Med. 261:894-895.

De Bord, G. G. 1942. Description of *Mimeae* tribe with three genera and three species. Iowa State J. Sci. 16:471-480.

Graber, C. D., W. T. Tumbusch, R. P. Rudnicki, and E. H. Vogel, Jr. 1960. Generalized Shwartzman-like reaction following *S. marcescens* septicemia in a fatal burn. Surg. Gynecol. Obstet. 110:443-450.

Schaub, I. G., and E. D. Hauber. 1948. A biochemical and serological study of a group of identical, unidentifiable gram-negative bacilli from human sources. J. Bacteriol. **56**:379-385.

Tumbusch, W. T., E. H. Vogel, Jr., J. V. Butkiewicz, C. D. Graber, D. L. Larson, and E. T. Mitchell, Jr. 1961. Septicemia in burn injury. J. Trauma **1**:22-31.

von Lingelsheim, J. 1905. Die bacteriologischen Arbeiten der Kgl. hyeienischen Station zu Benthen O-Schles während der Genickstarreepidemie in Oberschlesien im Winter. 1904-5. Klin. Jahrgb. Jena. **15**:373-488.

Weed, L. A. 1961. *Mimeae* - A problem in diagnosis and taxonomy. Am. J. Clin. Pathol. **35**:447-448.

# DEVELOPMENT OF IMMUNITY AGAINST TETANUS UNDER A VARIETY OF EXPERIMENTAL CONDITIONS

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Toxoid immunization against tetanus is today generally accepted to be both the most efficient and the most harmless of all vaccinations. From a theoretical point of view, we should be able to protect, and to keep protected for a lifetime, practically 100% of all immunized subjects. The only exceptions would be persons affected by the rare condition of agammaglobulinemia or by severe radioactive damage.

As a matter of fact, this hypothetical percentage of protected subjects is not generally reached. This is due to several reasons. First, not all of the basic immunological facts about the action of tetanus toxoid are sufficiently known. And where known, they have not always been taken into consideration. Especially, it must be remembered that not everyone having a history of tetanus immunization is actually immune. Actual immunity has not yet been carefully defined. Second, different types of toxoid are in use and their properties are not necessarily identical. This is especially true for fluid and adsorbed toxoids. Third, toxoid is often combined with the administration of horse antitoxin, and this combination raises special problems. Let me discuss these points one by one in the light of experimental evidence.

*Differing properties of toxoid.* The first toxoid injection in a patient does not provide immunity nor does it provoke an antibody response. The second injection has both of these effects, but they are not, under all circumstances, linked together. Actual protection against tetanus infection develops within 1 day, but the antitoxin response is detectable only after 3 to 4 days.

Thus, we may suppose that the actual "immune" status is reached shortly after the secondary toxoid stimulus or booster shot. The question which now arises, and the one that I have tried to answer is: how long does the status of actual immunity last and on which factors does its duration depend? It was soon realized that this question was closely tied to the type of toxoid.

*Choice of toxoid type.* It has been generally accepted that three injections of fluid toxoid are needed for immunization, although two

are considered sufficient if adsorbed toxoid is used. After two shots of fluid toxoid, not all subjects respond by antibody formation, but all subjects do respond to adsorbed toxoid.

### Methods

To study these conditions, antibody levels were evaluated over a period of 2 weeks to 6 years after two or three injections of fluid toxoid and after two or three injections of adsorbed toxoid.

### Results and Discussion

About 6 months after the second injection of adsorbed toxoid, 5 to 8% of the subjects presented no detectable antitoxin. This corresponded exactly to the results 2 weeks after the second injection of fluid toxoid. In other words, immunization by two doses of adsorbed toxoid provides statistical security only for a couple of months, and is therefore insufficient for long-term protection. Whether the third dose is considered as the completion of the basic immunization or as an early booster shot is a purely academic question. The important thing is to give it, as nearly as possible, 3 months after the initial injection. This interval has been shown to be the one of choice. After the third injection, in our sample, full protection lasted 2 years when adsorbed toxoid was used, and about 9 months when the fluid toxoid was used. A booster should be given to patients with wounds, if their vaccinations date back more than the above time intervals.

A second group of experiments concerned variations in the booster effect. These variations may not be of interest in countries where only adsorbed toxoid is used. In England and in several other countries, however, fluid toxoid is in use and therefore the following situations were studied: booster with fluid toxoid after basic immunization with adsorbed vaccine; both booster and immunization with fluid vaccine; adsorbed toxoid booster after vaccination by fluid toxoid; and adsorbed toxoid for all injections.

If adsorbed toxoid was used for the basic immunization, antitoxin response to the booster was rapid and the level was maintained for more than 1 year. The type of vaccine used for the booster shot did not measurably alter the results. On the other hand, in individuals originally vaccinated with fluid toxoid, the booster effect was delayed, and maintenance of the observed antitoxin level was significantly shorter.

From these experiments, one can conclude that adsorbed toxoid is to be preferred for basic immunization. For booster injection, any type of toxoid will do. The basic immunization and not the booster reaction (Table 1).

TABLE 1. *Response to fluid tetanus toxoid booster after various time intervals*

| Group* | Amount of antitoxin in serum† | Subjects responding | | |
|---|---|---|---|---|
| | | 2 to 6 months after booster | 1 year after booster | 2 years after booster |
| | | % | % | % |
| A | 0.02–0.1 | 14 | 20 | 50 |
| | 0.1–1 | 14 | 73 | 50 |
| | 1 | 72 | 7 | – |
| B | 0.02–0.1 | 8 | 10 | 16 |
| | 0.1–1 | 4 | 32 | 76 |
| | 1 | 88 | 58 | 8 |

*In group A, the basic immunization comprised three injections of fluid toxoid. The basic immunization of group B was with two injections of absorbed toxoid.

†International units per ml.

Development of immunity was also tested under conditions of simultaneous use of toxoid and serum. With the thought that there might be a basic difference if serum were given simultaneously with the first toxoid injection instead of with a subsequent one, the following experiment was performed. We had at our disposal a group of more than 700 humans, among whom some 50 had received (several years previously) 3,000 units of horse antitoxin and a single toxoid injection. For various reasons, their vaccination had never been completed. An antibody response in these individuals, after a single new toxoid injection, would prove that the primary toxoid stimulus was not overcome by the simultaneous serum injection. Without exception, these individuals showed a typical booster reaction (Table 2). On the other hand, serum given simultaneously with a booster shot strongly depressed antibody response. This result seems to show that simultaneous use of serum with a booster shot is definitely contraindicated. It may be useful, however, in unvaccinated

TABLE 2. *Response to tetanus toxoid booster of subjects immunized with one toxoid injection plus horse serum**

| Amount of antitoxin in serum† | Subjects responding | | |
|---|---|---|---|
| | Before booster injection | 4 days after booster injection | 11 days after booster injection |
| | % | % | % |
| 0 | 100 | 42 | – |
| 0.02–0.1 | – | 58 | 4 |
| 0.1–1 | – | – | 16 |
| 1–10 | – | – | 76 |
| 10 | – | – | 4 |

*Subjects had received one toxoid injection and 3,000 units of horse serum 1 to 6 years previously.

†International units per ml.

wound patients and when the treating physician wants to start vaccination without delay. This is a procedure which I would support very strongly.

In summary, I support toxoid vaccination with adsorbed toxoid, by three injections, with frequent booster injections and without use of serum in any individual who has previously received toxoid.

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# EXPERIENCE WITH *CANDIDA* RECOVERED FROM VENOUS BLOOD

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Superimposed infection by ubiquitous bacteria or yeasts frequently complicates prolonged antibiotic treatment of bacterial infection. Local infections with organisms of low virulence, such as *Candida*, may provoke few symptoms or signs. Invasion of the blood stream by such common secondary invaders as the gram-negative rods and staphylococci is usually heralded by an abrupt change in the clinical course. Our own observations are very limited but suggest that candidemia produces a similar picture in most patients but may be accompanied by few constitutional manifestations, and may disappear spontaneously after withdrawal of antibacterials.

A survey of the literature indicated that the most constant factor usually present prior to recognition of candidemia was antibiotic therapy. This, together with corticosteroids and debility due to bacterial infection, surgery, neoplasia, dysproteinemia, and endocrinopathies, appeared to increase susceptibility to candidiasis. The etiology was identified specifically by various criteria in most of the cases. Although *C. albicans* was the most frequent cause of candidemia, it was no more lethal than the other identified and unidentified species. The mortality of untreated patients was approximately 98%.

Treatment in one form or another was directed toward the candidemia in most of the reported cases. Little can be said about the relative effectiveness of the various agents used in treatment. Nystatin was used alone in five patients, four of whom recovered. Amphotericin B was administered to 17 patients, with a 53% mortality. It is apparent that early recognition and specific treatment may effect a cure in many instances.

In vitro sensitivity studies were reported for organisms isolated from seven patients, all of whom received amphotericin B. The minimal inhibitory concentration of amphotericin B required varied rather widely. All except two of the isolates were inhibited by concentrations of amphotericin well below that appearing in serum of patients receiving (intravenously) between 0.5 or 1.0 mg of amphotericin B per kg of body weight. Two strains of *C. albicans* required

2 μg/ml of amphotericin B for inhibition. This concentration of amphotericin is usually achieved or exceeded during and immediately following the intravenous infusion of recommended amounts of amphotericin.

There were many factors related to mortality, other than resistance to amphotericin or the amount and duration of therapy. The surgical removal of a focus of *Candida* may have been important to the outcome of the patient reported by Kay et al. (1961). In many instances, treatment failure seemed rather definitely related to delayed recognition and treatment and to the severity of the primary disease.

It is the purpose of this paper to present our experience with 13 cases of candidemia, in whom the clinical and laboratory observations were correlated in an attempt to learn: (i) possible criteria for early recognition of superimposed systemic candidiasis, (ii) the value of species identification of *Candida* and amphotericin sensitivity determination, (iii) indications for specific fungistatics in therapy, and (iv) results of treatment of severe candidemia with amphotericin B.

## Materials and Methods

With the exception of one infant, all cases were selected from the Louisiana State University School of Medicine services in the Charity Hospital of Louisiana at New Orleans. The patients were receiving treatment for various bacterial infections, and their unsatisfactory clinical course prompted the taking of blood cultures. Candidemia was found by serendipity in all of the early cases and many of the later ones. The number of cases presented does not necessarily reflect the incidence of *Candida* infection in our hospital. Diagnosis was established from broth inoculated with aseptically collected blood. When clinical findings suggested the specific possibility of yeast infection, small amounts of blood were inoculated into large volumes of brain heart infusion broth in an attempt to dilute any inhibitory substances which might be present in the serum. Broth cultures were examined for cloudiness of the fluid and for the appearance of small colonies in the sedimented blood. The broth flasks were observed for 2 to 4 weeks before being discarded. Subcultures were made onto blood agar plates and Sabouraud's medium. The Sabouraud cultures were incubated at room temperature and the others at 37 C. Specimens of urine, stool, and exudates were cultured in selected cases.

Species identification of the *Candida* isolated was made by the method advocated by Wickerham (1957, 1961).[1] Dalmau preparations were used for study of the morphological characteristics of aerobic and anaerobic growth. Carbon assimilation studies were done using

[1]The authors gratefully acknowledge the identification of *C. paropsilosis* by Lynferd Wickerham. These species were initially misidentified by us.

glucose, galactose, maltose, sucrose, trehalose, cellobiose, and raffinose. Fermentation studies were done as outlined by Wickerham with glucose, galactose, maltose, sucrose, lactose, and raffinose.

Sensitivity studies were done by incorporating the antifungal agent in Sabouraud's dextrose agar at pH 7. The tubes were slanted and rapidly hardened in the refrigerator. The surface of the slants was inoculated with 0.1 ml of a suspension of *Candida* calculated to contain 20 to 100 infective units. Each sensitivity series was in duplicate with duplicate controls. Slants were incubated at 27 C. The minimal inhibitory concentration of amphotericin was considered to be that amount which completely prevented growth of *Candida* on the first day of confluent growth in the control tubes.

## Results

A summary of clinical data preceding recognition of candidemia appears in Table 1. Ages ranged between 2 weeks and 64 years. Each patient had received at least three antibiotics, together or in sequence, prior to candidemia. All received penicillin and one of the broad-spectrum antibiotics. Between 8 and 213 days elapsed between the onset of antibiotic therapy and the appearance of candidemia.

*Candida* species were recovered from the urine of six patients, prior to appearance in the blood stream. One of these, patient 12, had *C. albicans* cultured from the bladder, right ureter, mouth, vagina, and stool, whereas candidemia subsequent to amphotericin therapy was due to *C. parapsilosis*. Candiduria was present frequently at the time of candidemia. However, patient 8 had a persistently sterile urine despite repeatedly positive blood cultures for *Candida*. Patient 10 had candiduria prior to, during, and following candidemia. The prolonged candiduria and azotemia, which subsided gradually during treatment with amphotericin B, suggested the presence of candidal pyelonephritis.

Patients 11 and 13 developed subcutaneous abscesses about the site of an intravenous infusion. Although the abscess in case 11 contained the same species of *Candida* as was isolated from the blood, patient 13 had *C. albicans* in the subcutaneous abscess and *C. guilliermondii* in the blood stream.

It is difficult to attach significance to the isolation of *Candida* from the saliva, sputum, or stool. The presence of clinical thrush or candidal vulvovaginitis seemed to be of some significance. Patients 1, 3, and 5 had clinical oral moniliasis prior to the appearance of candidemia. Patient 7 had decubitus ulcers containing *Candida* for many days prior to candidemia. These local infections may have served as the portal of entry for candidemia.

Evaluation of all possible signs of candidemia, as listed in Table 1, failed to lead us to any clinical syndrome whose constancy would

TABLE 1. *Summary of clinical data preceding recognition of candidemia* *

| Patient | Age | Primary disease | Pertinent therapy | | Day of disease Candida cultured from | | | Possible signs of candidemia |
|---|---|---|---|---|---|---|---|---|
| | | | Drug | Days | Blood | Elsewhere | | |
| | | | | | | Mouth | 14 | |
| 1 | 2 weeks | Septicemia, *S.albus* | SM | 15 | 22 | Urine | 22 | Persistent fever 102 F |
| | | | PEN | 15 | | | | Leukocytosis |
| | | | TM | 3 | | | | Debility |
| 2 | 12 years | Acute glomerulo-nephritis | PEN | 26 | 56 | None | | Sharp rise in fever 106F |
| | | | SM | 41 | | | | Lethargy, disorientation, coma |
| | | Septicemia, *A.aerogenes* | AM | 5 | | | | Hemorrhage: GI tract, conjuctivae, GU tract |
| | | | TM | 5 | | | | Enlarged kidneys |
| | | | Sulfa | 23 | | | | Uremia |
| 3 | 4 months | Agammaglobulinemia Furunculosis | PEN | 44 | 89 | Mouth | 24 | Recurrent fever 101F |
| | | | SM | 44 | | | | Leukocytosis |
| | | | TC | 30 | | | | Dermatitis |
| | | | EM | 6 | | | | Pneumonitis |
| | | | CM | 13 | | | | |
| | | | NOV | 7 | | | | |
| | | | MY | | | | | |
| | | | GG | 24 | | | | |
| 4 | 62 years | Diabetes mellitus | EM | 5 | 90 | Urine | 74 | Recurrent fever 101F |
| | | Subacute bacterial endocarditis | TC | 5 | | Urine | 79 | Leukocytosis |
| | | | Sulfa | 5 | | Urine | 86 | Lethargy |
| | | | PEN | 51 | | | | Pericarditis |
| | | | SM | 18 | | | | Congestive heart failure |
| | | | NOV | 8 | | | | Asthenia |
| | | | CM | 10 | | | | |
| 5 | 61 years | Diabetes mellitus | PEN | 8 | 44 | Urine | 31 | Persistent fever |
| | | Gangrenous foot | SM | 8 | | Stool | 34 | Leukocytosis |
| | | | TC | 7 | | Sputum | 35 | Meteorism |
| | | | PO | 3 | | Duodenum | 35 | Bloody diarrhea |
| | | | | | | Mouth | 37 | |
| 6 | 19 years | Subdural empyema | PEN | 33 | 28 | Urine | 13 | Sharp rise in fever 106F |
| | | | CM | 33 | 35 | Urine | 28 | Leukocytosis |
| | | | EM | 1 | | | | |
| | | | HC | 18 | | | | |
| 7 | 31 years | Meningitis | PEN | 17 | 90 | Skin | 40 | Recurrent fever 106F |
| | | | EM | 57 | | Urine | 85 | Leukocytosis |
| | | | CM | 68 | | | | Coma |
| | | | NOV | 36 | | | | Shock |
| | | | VA | 14 | | | | |
| | | | SPN | 47 | | | | |
| | | | TC | 7 | | | | |
| | | | BAC | 5 | | | | |
| | | | SM | 42 | | | | |
| | | | KA | 4 | | | | |
| | | | ACTH | 2 | | | | |
| | | | C | 2 | | | | |
| 8 | 6 years | Rheumatic heart disease | PEN | 213 | 225 | | | Recurrent septic fever to 104F |
| | | | CM | 36 | 226 | | | Leukocytosis |
| | | Acute endocarditis *S.aureus* | EM | 35 | 227 | | | Changing murmur |
| | | | TC | 16 | | | | |
| | | | VA | 16 | | | | |
| | | | SM | 15 | | | | |
| | | | C | 203 | | | | |
| | | | PEN | 41 | 356 | | | Recurrent intermittent fever to 104F |
| | | | C | 121 | 378 | | | Leukocytosis |
| | | | NOV | 40 | | | | |
| 9 | 21 years | Febrile puerperium | PEN | 16 | 30 | Cervix | 35 | Leukocytosis |
| | | | SM | 7 | 31 | | | Splenomegaly |
| | | | CM | 13 | 32 | | | Abrupt rise in fever, 105F |
| | | | EM | 6 | 34 | | | Pericarditis |
| | | | NOV | 5 | 35 | | | Disorientation |
| | | | | | | | | Hypotension |
| | | | | | | | | Congestive heart failure |
| | | | | | | | | Toxemia |
| 10 | 31 years | Septicemia *S.aureus* | PEN | 21 | 27 | Urine | 25 | Adynamic ileus |
| | | | CM | 26 | | | 27 | Disorientation |
| | | Renal failure | PO | 1 | | | | Jaundice |
| | | | HC | 4 | | | | Uremia |
| 11 | 64 years | Bacterial endocarditis *S.aureus* | PEN | 12 | 19 | Abscess | 32 | Fever spike |
| | | | CM | 6 | 20 | | | Leukocytosis |
| | | | EM | 9 | 22 | | | Thrombophlebitis |
| | | | | | 28 | | | Changing murmur |
| | | | | | 29 | | | Pericarditis |
| | | | | | 30-36 | | | Pleocytosis |
| 12 | 53 years | Pyelonephritis | Sulfa | 93 | 120 | Urine | 68 | Sharp rise in fever 105F |
| | | Hydronephrosis | PEN | 14 | | Stool | 90 | Leukocytosis |
| | | Paraplegia | SM | 8 | | Mouth | 90 | Disorientation |
| | | | CM | 16 | | Vagina | 90 | Shock |
| | | | FU | 38 | | | | Tachyardis |
| | | | KA | 11 | | | | |
| | | | PO | 4 | | | | |
| | | | Ampho | 16 | | | | |
| 13 | 17 years | Septicemia *A.aerogenes* | EM | 12 | 29 | Urine | 39 | Fever |
| | | | PEN | 8 | 42 | Urine | 43 | Leukocytosils |
| | | | CM | 14 | 46 | Wrist | 53 | Disorientation |
| | | | INH | 7 | | | | Pericarditis |
| | | | SM | 1 | | | | Pleuritis |
| | | | KA | 6 | | | | Myocarditis |
| | | | PO | 5 | | | | Shock |
| | | | HC | 5 | | | | |

*Key: AM=chlortetracycline, Ampho=amphotericin B, BAC=bacitracin, C=cortisone, CM=chloramphenicol, CO=colisti-methate, EM=erythromycin, FU=furadantin, HC=hydrocortisone, KA=kanamycin, MY=mystatin, NEO=neomycin, PEN=penicillin, PO=polymyxin, SM=streptomycin, Sulfa=sulfonamide, TC=tetracycline, TM=oxtetracycline, VA=vancomycin, GG=gamma globulin.

permit clinical diagnosis of candidemia. The signs and symptoms in most patients suggested either the persistence of bacterial infection or the appearance of superinfection of a type not etiologically recognizable on clinical grounds. Signs of toxemia resembling those seen in typhoid fever were present in those who had high fever, renal failure, acute cardiac failure, or central nervous system disease. Severe toxemia with mental depression, subsultus tendinum, and carphologia were present in patient 11 in the absence of fever. Pericarditis was seen in patients 4, 9, 11, and 13. Pericarditis may have been due to uremia in case 4 and *Aerobacter* sepsis in patient 13, but no etiology other than *Candida* was found for patients 11 and 9. It is interesting that patient 8 had no signs of debilitation despite hectic fever and repeatedly positive blood cultures for *Candida*. Lack of toxemia may have been due to the corticosteroids which she was receiving daily.

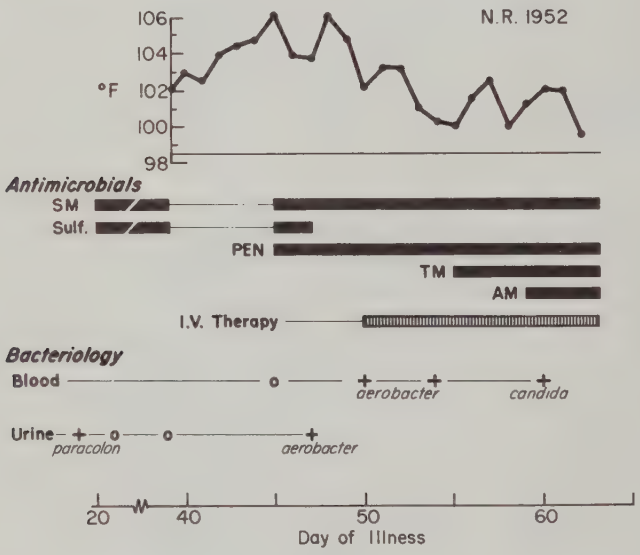


Figure 1

Three cases have been selected to illustrate the variable course of candidemia in patients treated or untreated for candidemia. Figure 1 depicts the temperature curve and pertinent data for a 12-year-old female whose candidemia was untreated. She had severe glomerulonephritis with bacterial urinary tract infection. The initial response to antibiotics was unsatisfactory, but fever increased when antibiotics were discontinued. Fever worsened on the 55th day of illness and was followed by coma, gastrointestinal hemorrhage, shock,

and death. Blood cultured 3 days prior to death was positive for *Candida*. Autopsy showed the immediate cause of death to be severe myocarditis with blastospores and pseudohyphae in the myocardium.

Figure 2 is a graphic illustration of the course of illness in a 6-year-old female under treatment for acute rheumatic fever. She was receiving corticosteroids and penicillin when *Staphylococcus aureus* endocarditis developed on the 205th day of her illness. The staphylococcal infection appeared controlled, but on the 225th day of illness, fever without toxemia appeared due to candidemia. Fever and candidemia disappeared within 3 days without specific treatment after antibiotics were changed. Candidemia reappeared 4 months later. The second episode was thought to be bacterial and antibiotics were reinstituted until *Candida* was isolated from the blood. Antibiotics were discontinued in the hope that *Candida* would again disappear. Fever and candidemia continued for 25 days, at which time it was decided to treat her with amphotericin B. Toxemia was noted after the institution of antifungal therapy and was associated with paracolon bacteremia. She responded well to treatment with chloramphenicol and kanamycin for 2 weeks and amphotericin B for 56 days.

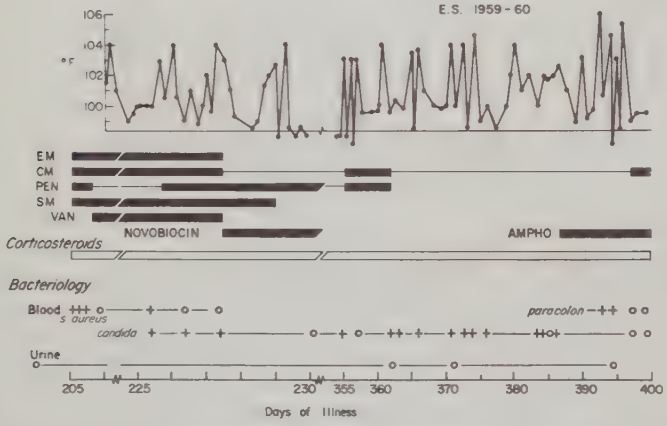


Figure 2

Figure 3 depicts the clinical course of patient 9, who underwent emergency Caesarean section after failure to replace a prolapsed cord. She was immediately febrile postoperatively, and was suspected of having staphylococcal endocarditis. She appeared to be well on the 30th day of intravenous treatment when she suddenly developed high fever, congestive failure, and disorientation. Leukocytosis, splenomegaly, and pericardial friction rub were noted. The latter

was confirmed by typical electrocardiographic changes of pericarditis. Blood cultures were positive for *C. parapsilosis* at this time. The cerebrospinal fluid was under increased pressure, contained an abnormal amount of protein, but was otherwise normal. The patient responded dramatically to the institution of treatment with amphotericin B and recovered uneventfully. It is possible that the cervix was the portal of entry.

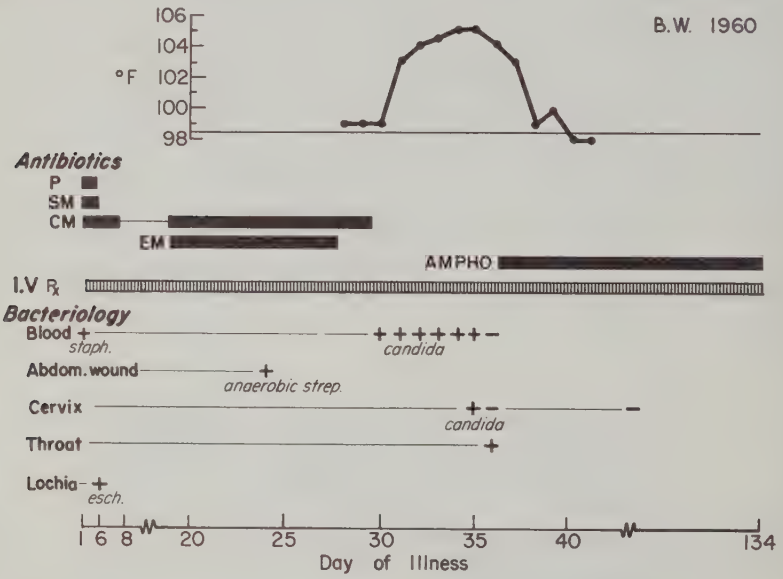


Figure 3

The most important facts relative to the course of our 13 patients with candidemia are summarized in Table 2. Five patients received no treatment for candidemia, and four of these died from their infection. Patient 6 had two episodes of candidemia, but recovered spontaneously when treatment with antibacterial antibiotics was altered. The same was true of patient 8 during the first bout of candidemia, but the second candidemia was persistent until treated with amphotericin.

Specific treatment of our patients consisted of the intravenous administration of amphotericin B in the manner previously described by Seabury and Dascomb (1958). Wherever possible, antibacterial antibiotics and adrenal corticosteroids were discontinued. Patients 8, 9, 10, 11, and 13 received presumably adequate treatment with amphotericin and recovered from candidiasis. Patient 12 apparently recovered from her initial candidiasis after relatively brief treatment

TABLE 2. *Summary of experience: patients with candidemia**

| Patient | *Candida* species | Involvement | Specific therapy | | | | | Sensitivity to amphotericin μg/ml | Comment |
|---|---|---|---|---|---|---|---|---|---|
| | | | Days after candidemia | Agent | Route | Duration, days | Amount | | |
| 1 | Not identified | Brain kidney lung liver spleen | - | none | - | - | - | - | Fatal, autopsy. |
| 2 | Not identified | Heart | - | none | - | - | - | - | Fatal, autopsy. |
| 3 | *albicans* | GI tract skin lung liver | (minus)4 | Ampho | I.V. | 26 | 0.126 | 2-0 | Death following severe reaction to Ampho. Sterile cultures obtained at autopsy. |
| 4 | *albicans* | Kidney endocardium pericardium spleen | 10 | Ampho | I.V. | 6 | 0.135 | 2.0 | Blood, urine sterile prior to death. Death due to congestive heart failure |
| 5 | Not identified | GI tract bone marrow lungs Kidney | - | none | - | - | - | - | Rapidly fatal. No autopsy. |
| 6 | *parapsilosis* | GU tract | - | none | - | - | - | 0.2 | Abruptly subsiding fever. Well 1.5 years since discharge |
| 7 | *tropicalis* | Skin GU tract | - | none | - | - | - | 0.2 | Fatal. Unrecognized *Candida* infection superimposed on tuberculous meningitis. No autopsy |
| 8 | Not identified | Endocardium | 2 | D/C antibiotics | - | - | - | - | Remission |
| | *parapsilosis* | | 24 | Ampho | I.V. | 58 | 1.269 | 0.4 | Prompt subsidence of fever. Recurrent fever due to paracolon bacteremia. Recovered |
| 9 | *parapsilosis* | Endocardium cervical aspirate GI tract urinary tract | 6 | Ampho | I.V. | 76 | 2.380 | 0.2 | Promptly subsiding fever and toxemia. Recovered |
| 10 | *tropicalis* | GU tract liver Lungs | 5 | Ampho | I.V. | 25 | 1.260 | 0.2 (urine) | Candiduria persisted 21 days after therapy. Fever persisted. Reaction to Ampho: hypokalemia, azotemia, thrombophlebitis. Recovered |
| 11 | *parapsilosis* | Endocarditis pericarditis thrombophlebitis subcutaneous abscess | 22 | Ampho | I.V. | 25 | 0.965 | 0.4 | Asthenia, candiduria and ECG changes resolved slowly. Recovered |
| 12 | *albicans* | GU tract GI tract | 26 | Ampho | I.V. | 16 | 0.415 | 0.2 (urine) | Promptly subsiding fever and disappearance of *Candida*. Blood remained sterile. |
| | *parapsilosis* | Blood | - | none | - | - | - | 2.-4.0 | Re-infection, sepsis, shock and death with Candidemia. |
| 13 | *guilliermondii albicans* | Blood Abscess pharynx GU tract | 17 | Ampho | I.V. | 60 | 2.1 | 2.-4.0 1.-2.0 | Recovered |

*Key: Sensitivity to amphotericin B determined for strain recovered from blood stream unless otherwise noted. – = Not pertinent. Ampho = Amphotericin B. I.V. = Intravenous. D/C = Discontinued.

with amphotericin. The terminal candidemia was untreated. Patient 4 did not receive sufficient amphotericin to evaluate its effect on his candidiasis, although both blood and urine became culturally sterile within 24 hr after amphotericin was started. Patient 3 was cured of his candidiasis by intravenous amphotericin but died from toxic effects of the drug.

If one excludes patient 4, who received only 6 days of treatment with amphotericin, the mortality was approximately 29% among the seven patients treated with intravenous amphotericin B. However, patient 12, who is included in the mortality figure, actually recovered

from the episode of candidiasis for which she was treated. The recovery rate is sufficiently better than that obtained (53% mortality) in previous reports to be encouraging, despite the small number of patients involved.

The preponderance of *C. parapsilosis* among our patients is so unusual that the possibility of nosocomial infection is suggested. This is a problem which requires further study.

## Discussion

Our experience with 13 patients with candidemia suggested nothing in the clinical picture which permitted differentiation from bacterial sepsis. The signs and symptoms associated with candidemia may be relatively mild or catastrophic. The variable clinical picture probably reflects differences in the host at the time candidemia appears, more than variation in virulence of the invading yeast. However, the fact that candidemia may disappear spontaneously with withdrawal or change of antibacterial antibiotics does suggest that opportunistic infection by *Candida* may at times be due to organisms of low virulence. This is in line with the experimental work of Mourad and Friedman (1961), who demonstrated considerable variation in the virulence of different strains of *C. albicans*. Recognition of candidemia depends solely on its isolation from blood cultures. Superinfection with fungi or bacteria should be suspected in any patient receiving prolonged antibacterial antibiotic treatment who has persistent fever or an abrupt worsening of the clinical course. Unless blood stream invasion by fungi is specifically kept in mind, blood cultures may be discarded before recognizable growth appears.

Our experience suggests that patients being treated with antibiotics and adrenal corticosteroids and who develop superficial or urinary tract infections with *Candida* are particularly likely to develop candidemia. This is especially true when such unfavorable host factors as debility, metabolic disease, or blood dyscrasia are present.

Species identification of *Candida* and the determination of in vitro sensitivity prove to be valuable in studying the epidemiology and pathogenesis of candidiasis. The unusually high incidence of *C. parapsilosis* in our patients suggests the possibility of nosocomial infection. Problems in the pathogenesis of candidemia are certainly suggested by the results of species identification of *Candida* recovered from patients 12 and 13. In patient 12, candidiasis was due to a different species than that which produced terminal candidemia. Patient 13 had a subcutaneous abscess due to a species of *Candida* different from that which had already produced candidemia.

Determination of the in vitro sensitivity of *Candida* isolates to amphotericin is easily done. The minimal inhibitory concentration

found may serve as a useful guide to daily dosage of amphotericin, particularly for those patients who have difficulty tolerating the antibiotic. There are in the literature an insufficient number of patients who have had sensitivity studies done during treatment with amphotericin to form any judgment concerning the utility of sensitivity studies in prognosis.

At the present time, it is our feeling that any patient with persistent candidemia who exhibits toxemia or definite worsening of the clinical course should be treated with intravenous amphotericin B. If the clinical picture associated with candidemia is not severe, the approach may be expectant if the antibacterial antibiotics being administered can be discontinued or basically altered without endangering the patient. It is apparent that some of these secondary invaders are of such low virulence as to disappear spontaneously when the conditions which have made possible their entry into the blood stream are removed. If candidemia does not disappear following withdrawal of antibacterial antibiotics and adrenal corticosteroids, treatment with amphotericin B probably should be instituted. Similarly, if the basic treatment regimen for the primary disease cannot be altered, candidemia *per se* is reason enough for instituting amphotericin therapy.

The duration of amphotericin therapy depends on the extent of disease if it can be estimated. Multiple abscesses and endocarditis deserve prolonged treatment. Surgical drainage of accessible collections of candidal pus is definitely indicated. In all cases, prolonged follow-up after treatment is necessary to detect recurrence.

## Literature Cited

Kay, J. H., S. Bernstein, D. Feinstein, and M. Biddle. 1961. Surgical cure of *Candida albicans* endocarditis with open-heart surgery. New Eng. J. Med. **264**:907-910.

Mourad, S., and L. Friedman. 1961. Pathogenicity of Candida. J. Bacteriol. **81**:550-556.

Seabury, J. H., and H. E. Dascomb. 1958. Experience with amphotericin B for the treatment of systemic mycoses. Arch. Internal Med. **102**:960-976.

Wickerham, L. J. 1957. Apparent increase in frequency of infections involving Torulopsis glabrata. Procedure for its identification. J. Am. Med. Assoc. **165**:47-48.

Wickerham, L. J. 1961. Taxonomy of yeasts. U. S. Dept. Agr. Tech. Bull. 1029. p.1-61.

# LABORATORY EVALUATION OF THE SPECIFIC SERUM ANTIMYCOBACTERIAL ACTIVITY TEST

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National Jewish Hospital (NJH) is presently involved in a cooperative clinical-laboratory investigation on the chemotherapy of patients with pulmonary tuberculosis caused by typical mammalian tubercle bacilli which are resistant to streptomycin and isoniazid. Using individually selected dosages and combinations of cycloserine, viomycin, kanamycin, ethionamide, and pyrazinamide, it has been possible to convert the sputum to negative in more than 90% of these patients within 120 days of the start of definitive chemotherapy. These results in "primary drug-resistant" patients with moderately- to far-advanced, bilateral, cavitary disease are extremely promising.

In addition to conventional drug susceptibility and 6-hr serum drug level determinations, these regimens have been monitored with a "specific serum antimycobacterial activity test" in which the patient's drug-containing serum is tested against his own strain of tubercle bacilli. This report presents the technical details regarding the performance of the test, data to illustrate the values obtained with different chemotherapy regimens, and a discussion of the factors which must be considered in the interpretation of test results.

## Materials and Methods

A detailed description of the patients and tuberculosis treatment regimens employed will be presented elsewhere. Patients were given graded test doses of each of the available secondary antituberculosis chemotherapeutic agents; 10-ml venous blood samples were collected aseptically 6 hr after the first morning dose of the drug. The blood was allowed to clot at room temperature, centrifuged, and the supernatant serum removed aseptically. Sterile blood samples were also obtained from each patient prior to drug therapy. Serums were stored in the deep freeze until tested.

After the selection and initiation of a definitive chemotherapy regimen, bloods were collected 6 hr after the first morning dose of the drug combination employed. Blood samples were obtained for testing at least once monthly during the period of drug treatment.

Prior to the initiation of definitive chemotherapy, one or more cultures of the *Mycobacterium* causing the disease was isolated from each patient by the standard methods devised at this hospital (Russell and Middlebrook, 1961). These cultures were maintained on egg-yolk agar and subcultured to Tween-albumin liquid medium prior to use.

The test was performed in six 13 by 100 mm, acid-cleaned, sterile glass test tubes with aluminum caps; 1 ml of oleic acid-albumin liquid medium, to which had been added 1% dextrose, was pipetted into each tube. Then 1 ml of the sterile serum to be tested was serially diluted through the first five tubes, to give final serum dilutions of 1:2 through 1:32. The sixth tube was a serum-free (and drug-free) control.

All six tubes were inoculated with 0.1 ml of a 14- to 28-day Tween-albumin liquid medium culture of the patient's own strain of *Mycobacterium*, shaken well, and incubated for 5 days at 37 C. At this time, 0.1 ml of a 1% aqueous solution of 2,3,5-triphenyltetrazolium chloride, sterilized by autoclaving, was added to all tubes. The tests were then shaken and incubated an additional 8 to 24 hr. Although tetrazolium reduction could be observed as early as 2 hr after the addition of the indicator, readings taken between 18 and 24 hr were used for the purpose of this paper.

The highest dilution of serum which inhibited reduction of the tetrazolium to the colored formazan was reported as the "specific serum antimycobacterial activity titer." Tests with no reduction in the 1:32 serum dilution were reported as "1:32 or higher"; those with reduction in the 1:2 dilution as "less than 1:2." Tests in which the indicator in the control tube was not reduced were obviously unsatisfactory.

In this laboratory, minor variations in technique did not cause significant changes in titer. Dilution of the inoculum by tenfold did cause an approximately twofold increase in inhibition titer; however, the standardization of inoculum size by turbidity or other means was not considered necessary for routine purposes.

Incubation periods of 3 to 10 days prior to addition of the tetrazolium indicator usually gave identical results; the decision to use a 5-day incubation period was an arbitrary one. Liquid cultures used for inocula, however, should be made in Tween-containing medium and should be from 1 to 4 weeks old. A few of the cultures incubated for less than 2 weeks were not fully grown. When cultures older than 4 weeks were used for the inoculum, the tetrazolium end points were unstable; prolonged incubation resulted in decreasing titers. End points were much more stable and reproducible with inocula prepared from 2- to 4-week cultures and seldom changed between 8 and 24 hr after the addition of the tetrazolium.

All strains of mycobacteria studied in this series were capable of reducing 2,3,5-triphenyltetrazolium chloride to the colored formazan. The addition of 1% glucose to the test medium resulted in earlier and more intense tetrazolium reduction.

*Correlation with clinical results.* During the initial phases of this study, the selection of drugs and dosage schedules was accomplished with the use of conventional drug-susceptibility studies and 6-hr serum drug levels. Specific serum antimycobacterial activity titers were performed after the administration of single and combined antituberculosis drugs; however, no attempt was made to correlate these test results with clinical findings until the first 30 patients had completed at least 90 days of definitive chemotherapy.

At this time, the "average serum inhibition titer" was calculated by adding the reciprocals of the available titers obtained during the initial period of definitive chemotherapy and dividing by the number of determinations. The value of 1:32 was used for those tests showing no tetrazolium reduction in the 1:32 serum dilution, even though the titer in these cases may have been considerably higher.

Individual patient average serum inhibition titers were plotted against the number of days of definitive chemotherapy required to produce the first of three or more consistently negative sputum cultures. This resulted in a "scatter graph" which showed the relationship between serum inhibition titers and the frequency of sputum conversion. Inspection of this graph provided a basis for the selection of a titer which was associated with consistent sputum conversion.

## Results

Prior to the administration of antituberculosis drugs, the serums of patients possessed little or no antimycobacterial activity under the conditions of this test (examples shown in Table 1). In no instance

TABLE 1. *Antimycobacterial activity titers of drug-free serums*

| Type of infection | Strain of *Mycobacterium* used as inoculum | | |
|---|---|---|---|
| | Patient's strain | *M. tuberculosis* H37Rv | *M. kansasii* |
| Human | < 1:2* | < 1:2 | 1:3 |
| Human | < 1:2 | < 1:2 | < 1:2 |
| Human | < 1:2 | < 1:2 | 1:2 |
| Human | 1:3 | 1:3 | 1:4 |
| Human | < 1:2 | < 1:2 | < 1:2 |
| Human | < 1:2 | < 1:2 | 1:3 |
| Human | 1:2 | 1:3 | 1:3 |
| Photochromogen | < 1:2 | < 1:2 | < 1:2 |
| Photochromogen | < 1:2 | < 1:2 | < 1:2 |
| Photochromogen | < 1:2 | < 1:2 | < 1:2 |
| Battey | < 1:2 | < 1:2 | 1:3 |

*No inhibition of tetrazolium reduction at a serum dilution of 1:2.

was a specific titer of more than 1:3 obtained. None of the patients with infections due to photochromogenic mycobacteria showed any inhibitory activity against their own strain of *M. kansasii* or against any other strain of *Mycobacterium* tested.

After the administration of single secondary antituberculosis agents, specific serum antimycobacterial activity titers were obtained which varied according to the susceptibility of the strain of tubercle bacilli being tested and with the nature of the drug (examples shown in Table 2). In general, titers were below 1:4 if the strain of *Mycobacterium* was resistant (by NJH criteria) to the drug being administered. If the organisms were susceptible to kanamycin or viomycin,

TABLE 2. *Specific serum antimycobacterial activity titers after administration of single antituberculosis drugs*

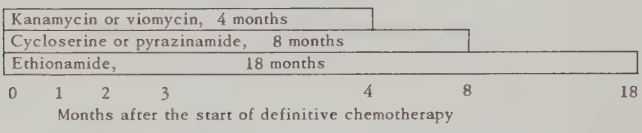
| Characteristics of strain of *Mycobacterium* used for inoculum | Drug | Dose | Route | Time (AM) | Six-hour serum level mg/ml (2:00 PM) | Specific serum antimycobacterial activity titer |
|---|---|---|---|---|---|---|
| Typical human, kanamycin-susceptible | Kanamycin | 20 mg/kg | i.m. | 8:00 | 11 | 1:12 |
| | | 10 mg/kg | | 8:00 | | |
| | | 5 mg/kg | | 11:00 | 15 | 1:12 |
| | | 10 mg/kg | | 8:00 | | |
| | | 10 mg/kg | | 11:00 | 32 | 1:32 |
| Typical human, kanamycin-resistant | Kanamycin | 20 mg/kg | i.m. | 8:00 | 5 | 1:2 |
| | | 10 mg/kg | | 8:00 | | |
| | | 5 mg/kg | | 11:00 | 8 | 1:2 |
| | | 10 mg/kg | | 8:00 | | |
| | | 10 mg/kg | | 11:00 | 12 | 1:2 |
| Typical human, cycloserine-resistant (50% growth on 10 μg/ml) | Cycloserine | 250 mg | oral | 8:00 | 22 | 1:2 |
| | | 500 mg | | 8:00 | 24 | 1:2 |
| | | 1000 mg | | 8:00 | 36 | 1:2 |
| Typical human, *p*-aminosalicylic acid-susceptible | *p*-Aminosalicylic acid | 4 g | oral | 8:00 | | |
| | | 4 g | | 12:00 | | 1:32 |
| Typical human, *p*-aminosalicylic acid-resistant | *p*-Aminosalicylic acid | 4 g | oral | 8:00 | | |
| | | 4 g | | 12:00 | | 1:2 |
| Typical human, ethionamide-susceptible | Ethionamide | 250 mg | oral | 8:00 | 4.5 | 1:32 |
| | | 500 mg | | 8:00 | 12.0 | 1:32 |
| | | 1000 mg | | 8:00 | 18.0 | 1:32 |
| Typical human, pyrazinamide-susceptible | Pyrazinamide | 250 mg | oral | 8:00 | | 1:4 |
| | | 500 mg | | 8:00 | | 1:4 |
| | | 1000 mg | | 8:00 | | 1:6 |
| *M. kansasii*, viomycin-susceptible | Viomycin | 20 mg/kg | i.m. | 8:00 | 14 | 1:10 |
| | | 10 mg/kg | | 8:00 | | |
| | | 5 mg/kg | | 11:00 | 20 | 1:12 |
| | | 10 mg/kg | | 8:00 | | |
| | | 10 mg/kg | | 11:00 | 28 | 1:16 |

titers as high as 1:32 or higher could be obtained with appropriate dosages of these agents.

For cycloserine or pyrazinamide given alone, titers were never higher than 1:8 and usually 1:4 or lower. This was also true for pyrazinamide when the test was performed at pH 5.6. For ethionamide in the dosages tested, the range of titers was from 1:4 to 1:32 or higher. With this drug, titers varied considerably from day to day in the same individual; increasing the test dose from 250 mg to 1 g failed to produce a consistent increase in serum activity. Prolonged administration of ethionamide appeared to result in lower serum activity titers with the same dosage, but this was difficult to confirm because of the variability in ethionamide serum activities within individuals.

Examples of specific activity titers in various phases of the tuberculosis treatment regimens are shown in Table 3. During the first 4 months of definitive chemotherapy, while kanamycin (or viomycin) was being given, the serum inhibition titers were relatively high (averaging 1:16 to 1:32 or higher). When kanamycin (or viomycin) was discontinued, these titers fell to 1:2 to 1:8. It was thought that this drop probably reflected a decrease in the efficiency of the re-treatment regimens. This was in part confirmed when three patients whose sputum cultures became negative during the 4 months

TABLE 3. *Specific serum antimycobacterial activity titers obtained during tuberculosis treatment regimens*


Kanamycin or viomycin, 4 months
Cycloserine or pyrazinamide, 8 months
Ethionamide, 18 months
0 1 2 3 4 8 18
Months after the start of definitive chemotherapy

| Patient | Type infection | Average specific serum antimycobacterial activity titers | |
|---|---|---|---|
| C.B. | Typical human | 1:32 | 1:4 |
| J.B. | Typical human | 1:32 | 1:3 |
| R.B. | Typical human | 1:32 | 1:2 (relapse) |
| V.B. | Typical human | 1:32 | 1:8 |
| R.C. | Typical human | 1:5 (relapse) | |
| L.C. | Typical human | 1:32 | 1:16 |
| E.C. | Photochromogen | 1:12 | 1:4 |
| H.G. | Typical human | 1:32 | 1:2 (relapse) |
| F.H. | Typical human | 1:32 | 1:6 |
| I.K. | Typical human | 1:32 | 1:8 |
| G.M. | Typical human | 1:32 | 1:3 |
| G.N. | Typical human | 1:32 | 1:3 |
| R.S. | Photochromogen | 1:10 | 1:4 |
| V.S. | Typical human | 1:30 | 1:2 |
| D.T. | Typical human | 1:24 | 1:3 |
| L.W. | Typical human | 1:32 | 1:8 (relapse) |

of kanamycin therapy became positive on culture within 1 to 3 months after kanamycin was discontinued.

The initial correlation between the serum inhibition titers and sputum conversion to negative in the first 30 patients to be treated on these regimens was as follows: 22 of 23 patients with average 6-hr serum titers of 1:16 or higher showed negative sputum cultures within 90 days after the start of definitive chemotherapy. The other patient became culture-negative on approximately the 130th day after the start of treatment. Of the seven patients with 6-hr serum inhibition titers of 1:10 or less, four were still culture-positive after 90 days. There were no patients in this initial series with average specific serum activity titers between 1:10 and 1:16 during the first 4 months of treatment.

Primary tuberculosis chemotherapy regimens employing streptomycin and isoniazid were also monitored with this test. In general, results appear to be similar but the clinical correlations are not yet available (Dye and Kass, *unpublished data).*

## Discussion

As a general rule, the large majority of effective chemotherapeutic agents used for the treatment of microbial infections act by direct inhibition or killing of the parasite in the tissues of the host. These drugs exert their effects in approximately the same range of concentrations in vivo as they do against the same organism growing in the test tube.

Theoretically, if the in vitro drug susceptibility of the parasite and the concentration of active drug delivered to the site in the lesion are known, the results of drug therapy should be predictable. In the infections considered in this paper, it is relatively easy to isolate the tubercle bacilli from the sputum of a given patient and to determine susceptibility to graded concentrations of the available antimicrobial agents. With the rare exception of tissues resected during surgery or at autopsy, it is practically impossible to estimate the approximate drug concentrations within the lesions where the tubercle bacilli are to be found. On the other hand, it is not difficult to obtain samples of blood from patients receiving chemotherapeutic agents and to test this blood or plasma for both the absolute concentration of drug present and for specific antimicrobial activity against the pathogen causing the infection in that individual.

It should be clearly recognized that the drug concentrations found in plasma and in tuberculous lesions are not necessarily equal, and that the plasma:tissue concentration ratio will vary markedly depending upon the nature of the lesion, the drug being administered, and the times at which the levels are being tested. Even so, the serum drug level can frequently be used as a rough index of the tissue

levels obtained and the concentration of antimicrobial agent available for delivery to the parasite. Logically, the plasma or serum drug level should be somewhat higher than that necessary to inhibit or kill the organisms within the lesions. Some concept of the variability of plasma:lesion ratios which may be expected to be required for effective chemotherapeutic action can be obtained from the data presented by Mackaness and Smith (1952) showing relative concentrations of drugs required to inhibit tubercle bacilli growth in Tween-albumin liquid medium and in the tissue culture medium surrounding macrophages in which the bacilli are growing intracellularly.

Naturally, the ultimate criterion governing the selection of an "adequate" serum drug level must be satisfactory therapeutic response. Clinical experience at NJH in the treatment of pulmonary tuberculosis with streptomycin and isoniazid suggests strongly that the achievement of 6-hr serum concentrations of these drugs approximately ten times that required for the in vitro inhibition of susceptible tubercle bacilli will consistently give satisfactory treatment results (Russell and Middlebrook, 1961). For the secondary antituberculosis drugs, similar clinical correlations are not yet available, but the ratios between the in vitro minimal inhibitory concentrations and achievable 6-hr serum levels appear to be of the same order as those known to be effective with streptomycin and isoniazid (Table 4).

TABLE 4. *Comparison of minimal inhibitory concentrations against susceptible typical mammalian tubercle bacilli and known effective or achievable 6-hour serum drug levels of selected antituberculosis chemotherapeutic agents*

| Drug | Approximate minimal inhibitory concentration | Effective or achievable 6-hr serum level | Ratio |
|---|---|---|---|
| | *μg/ml* | *μg/ml* | |
| Isoniazid | 0.04 | 0.4 | 1:10 |
| Streptomycin | 2 | 20 | 1:10 |
| *p*-Aminosalicylic acid | 1.5 | 30 | 1:20 |
| Kanamycin | 2 | 20 | 1:10 |
| Viomycin | 2 | 20 | 1:10 |
| Thioamide | 0.6 | 6 | 1:10 |
| Cycloserine | 5 | 20 | 1:4 |
| Pyrazinamide | 5 | 20 | 1:4 |

With this background, it appeared logical to assume that if the secondary antituberculosis drugs were being administered in adequate doses, the 6-hr serum samples obtained after these drugs were administered should be capable of inhibiting the growth of the individual patient's strain of tubercle bacillus. In addition, it seemed logical to assume that these sera should possess enough "acquired antimicrobial activity" to inhibit the tubercle bacilli when diluted at least 1:10 and possibly higher.

To initiate the tuberculosis re-treatment regimens, it was necessary to isolate and prepare broth cultures of the individual strains of tubercle bacilli isolated from the patients. Further, the serums obtained 6 hr after various test doses of single secondary drugs were already available from the drug level determinations necessary to "tailor" the chemotherapy needs of individual patients. Thus, it was relatively simple to prepare dilutions of these drug-containing serums in oleic acid-albumin liquid medium, add a small quantity of the broth culture of the patient's strain of tubercle bacillus, incubate the cultures, and observe the dilution of serum at which growth inhibition occurred. In practice, it was found that certain standardized conditions, including a 5-day incubation period, were necessary for reproducible results. Also, the addition of a small quantity of a 1% aqueous solution of 2,3,5-triphenyltetrazolium chloride at the end of the incubation period was a valuable aid in estimating the end point. After further overnight incubation, the bacilli in the control tube and in the presence of subinhibitory concentrations of the drugs reduced the tetrazolium indicator to the red formazan. In tubes in which the bacilli were inhibited or killed, the tetrazolium indicator remained colorless. The use of the oxidation-reduction indicator, tetrazolium, to show loss of bacterial viability as an index of drug action was suggested by the earlier work of other investigators (Vandiviere et al., 1961; Kubica and Vestal, 1960).

This "specific serum antimycobacterial activity test" is not an original concept. As early as 1918, Moore and Chesney used the "acquired bactericidal power of serum" against pneumococci to determine the best dosage schedule of ethylhydrocuprein for use in the treatment of pneumonia in man. More recently, Schlicter, MacLean, and Miller (1949) suggested the use of a similar procedure to monitor the chemotherapy of subacute bacterial endocarditis. Fisher (1952) demonstrated the value of testing the patient's drug-containing serum against his infecting organism in the treatment of acute bacterial infections. Fisher and Causton (1959) suggested essentially the same procedure as a guide to the treatment of tuberculosis, using serum containing the primary antituberculosis agents (streptomycin, isoniazid, and *p*-aminosalicylic acid). Kreis (1959) used a slide-culture method for this same purpose, again with the primary drugs. The major difference between these earlier papers and the method presented here lies in the use of the "specific serum antimycobacterial activity test" with strains of tubercle bacilli resistant to the primary antituberculosis drugs and tested against serum obtained after several graded doses of each of the available secondary drugs. In addition, the correlation between the activity levels and the therapeutic response as shown by sputum conversion should prove valuable, both as a guide to the use of these drugs and as a standard with which to judge the probable effectiveness and logical dosages of newer antituberculosis drugs as they become available for investigation.

The presence of antimycobacterial activity in low dilutions of the serum of patients and animals with tuberculous infections has been noted by others (Kochan and Raffel, 1960). The significance of this observation is not evident at this time. Under normal circumstances, this property of the serum should not be expected to interfere with the specific serum antimycobacterial activity acquired after the administration of chemotherapeutic agents.

Because of the known problems involved in the demonstration of antimycobacterial activity produced by pyrazinamide, no attempt should be made to interpret the results of these serum tests after administration of this agent. Performance of the test at lower pH's did not increase the serum titers in these experiments.

In accordance with established principles of antimycobacterial chemotherapy (Russell and Middlebrook, 1961), it is imperative that at least *two* effective drugs be administered concomitantly. An apparently satisfactory specific serum antimycobacterial activity titer observed with a combined drug regimen could conceivably result from the effects of a single effective agent. Theoretically, each of the drugs administered should demonstrate some level of serum activity when administered alone. In practice, single drug activity can be determined during the drug level studies prior to selection of the regimen, or by single drug administration for 1 or 2 days during the period of definitive chemotherapy.

From the high incidence of sputum conversion produced with these tuberculosis re-treatment regimens, it is obvious that "appropriate and adequate" secondary drug combinations can be selected by means of conventional drug susceptibility and 6-hr serum level determinations. Unfortunately, the laboratory procedure and technical personnel required for these determinations are not available in most tuberculosis bacteriology laboratories. The specific serum antimycobacterial activity test described in this paper may be performed with materials and equipment which are or should be available in every tuberculosis bacteriology laboratory. As additional experience and clinical correlations are obtained with the new test, it may provide a simple, rapid laboratory procedure which can be widely employed to aid in the selection and use of chemotherapeutic agents for the treatment of pulmonary tuberculosis.

## Acknowledgments

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## Literature Cited

Fisher, A. M. 1952. A method for the determination of antibacterial potency of serum during therapy of acute infections. Bull. Johns Hopkins Hosp. **90**:313-320.

Fisher, A. M., and J. Causton. 1959. A preliminary report on an antimycobacterial serum test. Bull. Johns Hopkins Hosp. **105**:360-369.

Kochan, I., and S. Raffel. 1960. A property of immune sera inhibitory for the growth of the tubercle bacillus. J. Immunol. **84**:374-383.

Kreis, B. 1959. Effectiveness of antituberculous treatments tested by direct culture of the bacilli in the patient's blood. Am. Rev. Respiratory Diseases **80**:85-88.

Kubica, G. P., and A. L. Vestal. 1960. A rapid drug-susceptibility test for mycobacteria: A preliminary report. Am. Rev. Respiratory Diseases **83**:593-595.

Mackaness, G. B., and N. Smith. 1952. The action of isoniazid (isonicotinic acid hydrazide) on intracellular tubercle bacilli. Am. Rev. Tuberc. **66**:125-133.

Moore, H. F., and A. M. Chesney. 1918. Study of ethylhydrocuprein (optochin) in the treatment of acute lobar pneumonia. Arch. Internal Med. **21**:659-681.

Russell, W. F., Jr., and G. Middlebrook. 1961. Chemotherapy of tuberculosis. Charles C Thomas, Springfield, Ill.

Schlicter, J. G., H. MacLean, and A. Miller. 1949. Effective penicillin therapy in subacute bacterial endocarditis and other chronic infections. Am. J. Med. Sci. **217**:600-608.

Vandiviere, H. M., C. E. Rogers, I. G. Melvin, and H. S. Willis. 1961. A rapid method of testing the susceptibility of mycobacteria to antituberculous drugs. Am. Rev. Respiratory Diseases **84**:399-406.

# SPECIFIC SERUM ANTIMYCOBACTERIAL ACTIVITY TEST AS A GUIDE TO EFFECTIVE ANTIMYCOBACTERIAL THERAPY

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An urgent problem in the fight against tuberculosis is the worldwide rise in the number of patients excreting *Mycobacterium tuberculosis* resistant to the primary antituberculosis drugs (Jancik et al., 1961). The problem has become even more threatening since repeated clinical studies have demonstrated that the so-called "secondary" agents do not possess the therapeutic efficiency observed with the "primary" drugs, streptomycin (SM) and isoniazid (INH) (Moyer and Schwartz, 1957; Birkeland and Nissen-Meyer, 1958).

The satisfactory results obtained with the primary drug regimens at this hospital (Kass et al., 1957) have encouraged the development of similar treatment protocols utilizing the available secondary drugs. Using a combination of cycloserine (CS) and pyrazinamide (PZA) or kanamycin (KM), or both, a reversal of sputum infectiousness was achieved in 17 (36%) of 37 patients who were excreting *M. tuberculosis* that were resistant to SM and INH (Haapanen et al., 1960). The over-all salvage rate was increased to 64% with drugs plus surgery.

Unfortunately, this earlier study gave rise to certain disappointments: First, the findings were too inconclusive to base any valid judgment concerning the therapeutic efficacy of the various regimens on them; second, there was no way of predicting individual patient response to the drug regimen, for treatment failure sporadically occurred after months of apparently successful chemotherapy.

In December 1960, a new re-treatment protocol was initiated, using 45 primary drug-resistant patients. This investigation differed from the earlier study in the administration of kanamycin; ethionamide was included in the chemotherapy regimens; and the specific 6-hr serum antimycobacterial activity test (Dye, Kass, and Gill, 1962) was used to monitor the effectiveness of these secondary drugs. The purposes of this paper are to summarize the findings regarding the patients' responses to the chemotherapy regimens and to demonstrate the value of this specific testing procedure as a guide to effective antimycobacterial therapy in tuberculosis.

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## Materials and Methods

Forty-five patients with pulmonary tuberculosis were selected according to the following criteria at the time of the initiation of treatment. They were known to be: (i) excreting typical mammalian tubercle bacilli resistant to INH (0.2 μg/ml), SM (2 μg/ml), and usually also to *p*-aminosalicylic acid (PAS) (3 μg/ml); (ii) sputum-positive on two consecutive cultures within a fortnight prior to the start of treatment; (iii) excreting organisms susceptible to ethionamide (E) (5 μg/ml), KM, or viomycin (VIO) (5 μg/ml), and either CS or PZA (both in concentrations of 10 μg/ml); and (iv) suffering from active bilateral pulmonary disease with at least one cavity manifest on the chest roentgenogram.

In 26 patients, chest roentgenograms showed disease classified as far advanced; in 19 patients the roentgenograms indicated disease in the moderately advanced stage.

No therapy was initiated until it had been ascertained that the patient's organisms were susceptible to the antimycobacterial agents to be used, and that an effective serum level for each drug could be obtained with the dosages employed.

At National Jewish Hospital, populations of tubercle bacilli are considered to be susceptible to E, KM, VIO, CS, or PZA if on direct testing on 7H-10 solid medium, less than 1% of the number of the organisms yielding colonies on the control can grow on the same culture medium containing 5 μg/ml of E, 5 μg/ml of KM, 5 μg/ml of VIO, 10 μg/ml of CS, or 10 μg/ml of PZA (the PZA studies are done separately since the culture medium has to be maintained at a pH of 5.6).

The serum for the 6-hr microbiological assays for each of the secondary drugs was collected on three consecutive days. For KM or VIO the loading dose on the first day was 1 g given in a single injection. On the second day, the patient received 10 mg/kg, of either KM or VIO, followed by a 5 mg/kg dose given 3 hr later. On the third day, the second injection was increased to 10 mg/kg. For E and CS the loading dose on the first day was 250 mg; on the second day it was 500 mg; and on the third day it was 1,000 mg. No practical methods of determining serum levels were available for PZA. For each individual patient the 6-hr serum levels achieved with E and CS were practically the same, regardless of the loading dose; therefore, there appeared to be no need to administer more than 250 mg of these agents four times a day. Since no PZA serum levels were available, all but the physically smaller patients each received 3 g of this medication daily. The dosage for each in the latter group was 2 g per day. At least two, and preferably three, secondary drugs were prescribed; the following dosage schedules were used: (i) Ethionamide (uncoated). The dosage was oral, either 250 mg three times a day (8:00 AM, 10:00 AM, and 12:00 noon), or 250 mg

four times a day (8:00 AM, 10:00 AM, 12:00 noon, and 4:00 PM). Patients unable to tolerate oral E were given 500 mg rectal suppositories twice a day. Therapy with this drug was scheduled for a period of 18 months. (ii) Kanamycin or viomycin. The dosage schedule for both drugs was 10 mg/kg intramuscularly at 8:00 AM, and either 5 or 10 mg/kg at 11:00 AM, 5 days a week for 4 months. (iii) Pyrazinamide or cycloserine. If pyrazinamide, the dosage was 3 g daily, orally (1 g administered at 8:00 AM, and 1.0 g at 12:00 noon; 0.5 g prescribed for 10:00 AM, and 0.5 for 4:00 PM). If cycloserine, 250 mg, orally (8:00 AM, 10:00 AM, 12:00 noon, and 4:00 PM). Patients requiring CS also received orally 300 mg of pyridoxine and 1,200 mg of meprobamate daily. Both CS and PZA were given for a period of 8 months.

The regimen described above was monitored by a "specific serum antimycobacterial activity test" done prior to the initiation of definitive chemotherapy and repeated at least once monthly. This serum study was performed as described in the accompanying paper (Dye et al., 1962).

Before definitive chemotherapy was begun, each patient was subjected to a baseline chest radiograph, including tomograms if indicated; susceptibility studies for INH, SM, PAS, KM, VIO, E, PZA, Cs, and dextro-2,2'-(ethylenediimino)-di-1-butanol (Ethambutol, Lederle); a complete blood count, including erythrocyte sedimentation rate; routine urinalysis; liver function tests, including serum glutamic oxalacetic transaminase (SGOT), serum glutamic pyruvate transaminase, Bromsulphalein, thymol turbidity, serum bilirubin, alkaline phosphatase, and prothrombin time; and an audiometric survey.

During chemotherapy, the patient was subjected to a monthly chest roentgenogram; studies of biweekly sputum specimens for smear and culture for acid-fast bacilli and succeptibility to the particular drugs being used in this regimen; a monthly study of SGOT and determination of serum uric acid (for the patients receiving PZA). If the SGOT were elevated, a complete battery of liver function tests was ordered before any changes in the therapy were instituted. Audiograms were obtained monthly. Routine urinalysis, blood urea nitrogen, and phenolsulfonphthalein renal excretion determinations were ordered every 2 weeks for those patients receiving VIO.

As soon as three successive negative sputum cultures for *M. tuberculosis* were recorded, the patient's case was presented to the surgical conference. Whenever indicated, thoracic surgical procedures were performed without interrupting the planned course of chemotherapy.

## Results

At the time of this report, 45 patients had completed at least 90 days of definitive chemotherapy on the secondary drug regimen;

43 of these 45 patients (95%) became sputum-negative for *M. tuberculosis* on at least three consecutive sputum examinations.

In the patients who became sputum-negative, the average duration of chemotherapy, prior to the first negative sputum culture, was 53 days; with the exception of the four patients who relapsed, the sputa of these patients have remained negative on culture for an average of 105 days. The average length of chemotherapy at the time of this analysis, was 166 days. Seventeen patients had resectional surgery on an average of 86 days after sputum conversion to negative. One patient had bilateral surgery in which both upper lobes were removed. In every instance, surgery was used to maintain the sputum negativity, rather than to achieve it. There was no immediate postoperative morbidity.

Thus far, sputum relapses have been observed in four cases. In three patients, this occurred 26, 71, and 85 days, respectively, after treatment with KM was discontinued. The fourth patient became sputum-positive while KM was still being administered.

In 41 patients, the average specific serum antimycobacterial activity titer recorded during the first 4 months of chemotherapy was greater than 1:16. Two patients with a titer greater than 1:16 did not become sputum-negative; two patients required more than 120 days to do so. Thus, 37 of 41 (90%) patients became sputum-negative within the 4 month period. The sputum of three patients in this group again became positive for *M. tuberculosis* after KM was discontinued.

Of the four patients who had achieved a serum activity titer of less than 1:16, one patient required more than 120 days for sputum conversion. There was one relapse to a sputum-positive condition in this group. One patient with a low serum activity titer was treated with CS, PZA, and E, but not KM, because of renal insufficiency due to an earlier nephrectomy.

When KM was included in the drug combination, the average serum antimycobacterial activity titer was 1:27; when the KM was discontinued, the titer fell to 1:8. When VIO was substituted for KM, there was no change in the titer.

In a separate study comprising 27 different patients, the average specific serum antimycobacterial activity titer obtained when the patients were treated with E, PZA, or CS was 1:8; when KM was added to these drugs, the titer rose to an average of 1:30. Toxic reactions to the drugs were observed in 20 patients.

*Kanamycin.* One patient became deaf; a marked hearing loss was noted in another. (These two patients had previously received a protracted course of SM and had significant auditory damage prior to the initiation of KM therapy.) Two patients had a moderate hearing loss; a mild hearing defect was detected in another. One individual complained of symptoms of subjective vertigo; two others experienced annoying sensations of aural fullness as well as tinnitus.

In addition, many patients had an objective loss of hearing acuity in the higher frequencies; the KM, however, was not discontinued, because the audiometric measurements indicated that the lower frequencies, more important to daily communication, were not involved.

*Cycloserine.* Two patients were disturbed by a feeling of extreme nervousness while receiving CS; a third individual developed a marked but reversible psychotic reaction. One patient displayed a marked photosensitivity involving the dorsal surfaces of both hands; this reaction disappeared completely when CS was discontinued.

*Pyrazinamide.* Although a slight rise in the serum uric acid level was observed in almost all of the patients receiving PZA, it was felt necessary to treat only one patient with a uricosuric drug.

*Ethionamide.* Although many of the patients receiving E had gastrointestinal discomfort, especially after the start of therapy, these symptoms usually diminished after 2 to 3 weeks of therapy. In three patients this gastrointestinal disturbance was sufficiently severe to necessitate a change from oral to the suppository form of the preparation. Three patients developed a skin rash while receiving E, and another individual suffered from a postoperative depression.

Of the six patients who remained either persistently sputum-positive or suffered a relapse to that state from an original conversion to negative, three had bacilli that were still susceptible to E and KM; but only one of the six patients had organisms remaining susceptible to CS.

## Discussion

As compared with previous experiences at this hospital, the increased success attained with these new secondary drug regimens may be attributed to the three factors discussed below.

*Dosage schedule for administering KM.* It had been found in previous investigations at this hospital that 1 g of KM given once a day, 5 days a week, for 4 months was inadequate, because 32 of 35 patients continued to excrete organisms susceptible to KM (Haapanen et al., 1960). Also, when 1 g of KM was given in a single dose, only 5% of the patients achieved a 6-hr serum level of microbiologically active KM that was greater than 20 $\mu$g/ml. The 6-hr serum level of microbiologically active KM was greater than 20 $\mu$g/ml in over 50% of the patients when 10 mg/kg of the drug was given twice a day, 3 hr apart. An associated rise in the specific serum antimycobacterial activity test was observed with this dosage schedule (Kass and Dye, *unpublished data).*

*Addition of ethionamide to the chemotherapy regimen.* The initial clinical trials of this drug at National Jewish Hospital were discouraging. The gastrointestinal symptoms were most disturbing to

many of our patients, and cultures of the sputa revealed no improvement. However, ethionamide was reinstated into the chemotherapy regimens after Brouet et al. (1959) and Somner (1959) reported that an effective antimycobacterial action could be demonstrated when E was administered in combination with other antituberculosis drugs. As the usage of the drug in this study increased, it was observed that the gastrointestinal symptoms were not as severe as anticipated and usually disappeared after 2 or 3 weeks of continuous therapy.

Studies indicate that E does not produce the same specific serum antimycobacterial activity titer that KM does. These studies also suggest that the antituberculosis action of this drug falls somewhere between INH and either CS or PZA. The "crowding" of the dosage of E into a 4-hr period (250 mg at 8:00 AM, 10:00 AM, and 12:00 noon) did not produce a statistically significant rise in the specific serum antimycobacterial activity titer (Kass and Dye, *unpublished data*). Since it is planned to continue therapy with E alone for a period of at least 18 months, a lengthy period of follow-up of present subjects is needed before any definite decisions may be made concerning the therapeutic efficacy of this drug.

*Application of the specific serum antimycobacterial activity test.* Although still in the developmental stage, this test has been of considerable help in determining and predicting the adequacy of the chemotherapeutic regimens. As soon as definitive chemotherapy with the secondary drugs, i.e., E, KM, and CS or PZA, is started, the serum is collected, as described, for the serum activity study (Dye et al., 1962). If a 1:16 or greater dilution of the patient's serum inhibits the growth of *M. tuberculosis*, therapy is continued; the existing data indicate that the patient has a 90% chance of becoming sputum-negative within 120 days.

Even though two of the patients did not become sputum-negative, despite a greater than 1:16 specific serum antimycobacterial activity titer, while four patients with serum activity titers of less than 1:16 did, the necessity of adjusting these chemotherapy regimens so that at least a 1:16 6-hr serum activity titer is attained increases when it is noted: first, that an average specific serum antimycobacterial activity titer of 1:32 was recorded when patients excreting *M. tuberculosis* susceptible to SM and INH were treated with these drugs; and second, three of the four patients who relapsed to the sputum-positive stage did so after the stoppage of the KM therapy had reduced their serum activity titer below the 1:16 level.

Despite the fact that surgery for the patients with pulmonary tuberculosis, who have been adequately treated with the primary drugs, is no longer advocated (Kass et al., 1960), the authors' experience with the secondary drugs is too limited to support a recommendation that these patients be treated solely by chemotherapy. Whenever possible, patients receiving secondary drugs at National

Jewish Hospital are operated upon as soon as their sputum becomes negative for *M. tuberculosis* on at least three consecutive cultures.

The incidence of significant toxic reactions associated with the use of E, KM, CS, or PZA was not high enough to contraindicate such chemotherapy.

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## Literature Cited

Birkeland, K., and S. Nissen-Meyer. 1958. Pyrazinamide and cycloserine in the treatment of pulmonary tuberculosis. Acta Tuberc. Scand. **36**:98-109.

Brouet, G., J. Maeche, N. Rist, J. Chevallier, and G. Lemeur. 1959. Observations on the antituberculous effectiveness of alpha-ethyl-thioisonicotinamide in tuberculosis in humans. Am. Rev. Respiratory Diseases **79**:6-18.

Dye, W. E., I. Kass, and R. Gill. 1962. Laboratory evaluation of the specific serum antimycobacterial activity test. Antimicrobial Agents and Chemotherapy – 1961, p. 20-29.

Haapanen, J., R. Gill, W. F. Russell, Jr., and I. Kass. 1960. Re-treatment of pulmonary tuberculosis; experiences with various combinations of pyrazinamide, cycloserine, and kanamycin in patients excreting tubercle bacilli resistant to both streptomycin and isoniazid. Am. Rev. Respiratory Diseases **82**:843-852.

Jancik, E., N. W. Horne, B. Kreis, M. Lucchesi, J. W. Raleigh, and M. Zierski. 1961. The treatment of pulmonary tuberculosis in patients harbouring bacilli resistant to the three major drugs (Symposium, September 8, 1960. Paris). Bull. Intern. Union Against Tuberc. **31**:3-60.

Kass, I., W. F. Russell, Jr., A. Heaton, T. Miyamoto, G. Middlesbrook, and S. H. Dressler. 1957. Changing concepts in the treatment of pulmonary tuberculosis. Ann. Internal Med. **47**: 744-761.

Kass, I., T. Miyamoto, J. Denst, G. J. Wittenstein, W. F. Russell, Jr., G. Middlebrook, and S. H. Dressler. 1960. The residual lesion in pulmonary tuberculosis requiring surgery: A review of 100 sputum-negative patients consecutively operated on. New Engl. J. Med. **262**:315-320.

Moyer, R. E., and W. S. Schwartz. 1957. Pyrazinamide and cycloserine in the treatment of pulmonary tuberculosis. Trans. 16th Conf. Chemotherapy Tuberc., VA-Armed Forces, p. 59-61.

Somner, A. R. 1959. 2-Ethylisothionicotinamide ('1314') in pulmonary tuberculosis: A controlled trial of drug tolerance. Tubercle **40**:457-461.

# BACTERIAL ENDOCARDITIS: THE CHANGING PATTERN SINCE THE INTRODUCTION OF PENICILLIN THERAPY

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Recent reports (Finland, Jones, and Barnes, 1959; Rogers, 1959) have reviewed the changing pattern of microbial disease in hospitalized patients since the advent of antibiotic therapy. These two studies have shown a striking decrease in incidence of serious infections caused by *Diplococcus pneumoniae* or *Streptococcus pyogenes* and a marked increase in systemic infections due to gram-negative enteric bacilli. One report (Finland et al., 1959) demonstrated an increased incidence of severe staphylococcal infections, but the other (Rogers, 1959) did not show this trend. Both reports concluded that microorganisms relatively resistant to the commonly used antibiotics have been responsible for an increasing proportion of serious bacterial infections encountered in hospitalized patients in recent years.

The present investigation was undertaken to study changes in patient population and etiologic microorganisms associated with bacterial endocarditis in a series of 383 cases observed at The New York Hospital during a 29-year period.

## Materials and Methods

Patients with bacteriologically confirmed endocarditis were included in the present study. During the 29-year period (1932 to 1960) two cases of fungal endocarditis were observed and are included in the series. The criteria for selection were as follows: (i) isolation of the same microorganism from two separate blood specimens from a patient with a syndrome consistent with bacterial endocarditis, (ii) isolation of a microorganism from a single blood culture during life, in addition to autopsy confirmation of the diagnosis, or (iii) isolation of a microorganism from vegetations or blood obtained from the heart at autopsy, in addition to presence of microorganisms on microscopic examination of the vegetation. These

strict criteria provided assurance of a correct diagnosis and eliminated questionable cases from the study. Patients with sterile blood cultures who were treated for bacterial endocarditis, without verification of the diagnosis by bacteriological methods at autopsy, were not included and will form the basis of a separate report.

To permit valid comparisons of the incidence of various underlying cardiac diseases during the period of this study, well-defined diagnostic criteria were established. Patients were considered to have rheumatic heart disease if a history of acute rheumatic fever was available, a murmur of mitral stenosis antedated the onset of bacterial endocarditis, mitral stenosis was demonstrated at the time of cardiac surgery, or classical findings of rheumatic heart disease were present at autopsy. The murmur of mitral stenosis noted after onset of bacterial endocarditis, in the absence of a history of rheumatic fever, was not considered sufficient evidence to diagnose rheumatic heart disease. Diagnosis of congenital heart disease was established by the history of a murmur from birth, presence of murmurs diagnostic of a congenital cardiac lesion, angiocardiography, cardiac catheterization, cardiac surgery, or autopsy. Arteriosclerotic heart disease was considered to be the underlying lesion only if postmortem examination revealed vegetations on a portion of the endocardium involved in the arteriosclerotic process. A diagnosis of no underlying heart disease was made when a history showed no cardiac murmurs prior to onset of endocarditis or when absence of underlying heart disease was documented at autopsy.

Autopsies were performed on 83 of the 173 patients observed from 1932 to 1943 and on 56 of the 210 patients seen from 1944 to 1960.

Cure was defined as survival with sterile blood cultures for at least two months after cessation of definitive antimicrobial therapy. Therapy was considered to have failed if death occurred within the two-month period, even if autopsy revealed healing, inactive bacterial endocarditis. Relapses of endocarditis caused by the same microorganism were counted as a part of the original episode, and success or failure of treatment was judged by the final outcome of the episode.

The patients observed from 1932 to 1943 were compared with patients admitted from 1944 to 1960. This division seemed pertinent as only one patient was treated with penicillin before 1944 and the vast majority of patients was subsequently given penicillin as part of the therapeutic regimen.

Penicillin susceptibility of microorganisms was determined by an antibiotic-dilution technic in broth.

## Results and Discussion

*Yearly incidence.* A total of 383 episodes of bacterial endocarditis was observed in 373 patients hospitalized at The New York Hospital from 1932 to 1960. During this 29-year period there were 577,449 admissions to the hospital, excluding newborns and admissions to psychiatry, giving an endocarditis incidence of 0.66 per 1,000 hospital admissions. A progressive decrease in number of cases of bacterial endocarditis per 1,000 admissions has occurred during the past 16 years (Fig. 1).

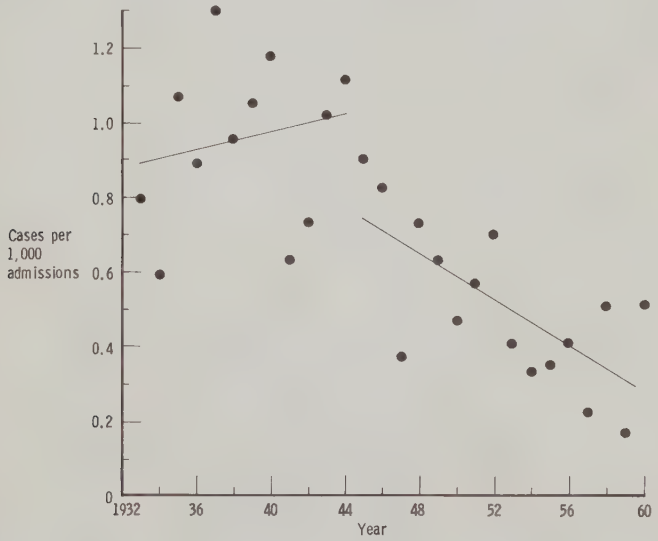


FIG. 1. *The incidence of bacterial endocarditis expressed as number of cases per 1,000 admissions to The New York Hospital. Slopes for 1933 to 1944 and 1945 to 1960 were calculated by the method of least squares. The lines shown pass through the mean year and mean case rate for each period.*

This decrease in incidence of bacteriologically-proven endocarditis, since the advent of antibiotic therapy, is not a unique experience and has been noted by others (Littmann and Schaaf, 1950; Angrist and Marquiss, 1954). A number of factors may have contributed to the decreased incidence, including widespread use of antibiotics in the treatment of infections of all types, appropriate use of antibiotics to prevent transient bacteremia in the susceptible host, or even decrease in number of patients with endocarditis referred to medical centers since successful antibiotic therapy became possible in community hospitals.

*Sex and age incidence.* The 383 episodes of endocarditis occurred in 220 males and 163 females. There was no striking difference in ratio of males to females between the 1932 to 1943 and 1944 to 1960 groups.

The age distribution of patients with bacterial endocarditis is shown in Fig. 2. These data demonstrate a significant shift ($P<0.01$) in age distribution during the two periods. The mean age of 103 males in the earlier series was 37 years with 42% over 40 years, whereas, in the later period the mean age of 117 males was 46 years with 62% over 40. The mean age of 70 females in the 1932 to 1943 series was 30 years with 23% over 40 and in the 1944 to 1960 series the mean age of 93 females was 40 years with 43% over 40.

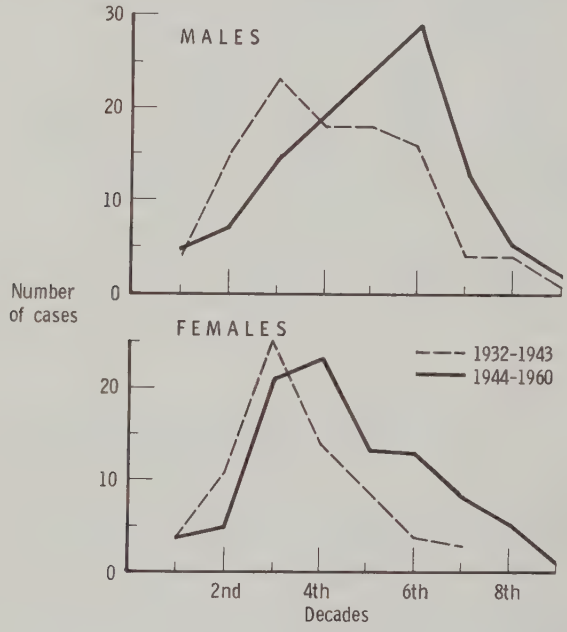


FIG. 2. *Distribution of age, by decades, of 383 patients with bacterial endocarditis.*

Comparison of two periods (1944 to 1951 and 1952 to 1960) within the 1944 to 1960 interval also revealed a similar trend of increasing age for both sexes.

The increasing proportion of older patients in the later series cannot be explained by an increase in number of cases of endocarditis caused by streptococci of the enterococcus group, which affects

an older male population (Robbins and Tompsett, 1951; Koenig and Kaye, 1961), or to an increased incidence of staphylococcal endocarditis. As shown in Fig. 3, a similar significant ($P<0.01$) trend was observed in patients with endocarditis caused by streptococci of the viridans group (Fig. 3).

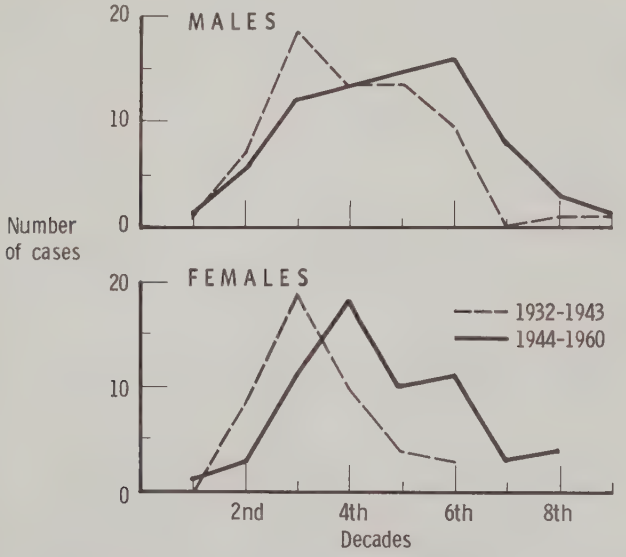


FIG. 3. *Distribution of age, by decades, of 248 patients with bacterial endocarditis caused by streptococci of the viridans group.*

The change in age distribution of patients with bacterial endocarditis has been described by other investigators (Cummings et al., 1960; Anderson and Staffurth, 1955; Hartman and Myers, 1959; Friedberg, Goldman, and Field, 1961) and may be partially explained by a longer life span of the population at large, and a longer life expectancy of cardiac patients who have had the benefit of modern medical or surgical therapy.

*Underlying heart disease.* Comparison of the incidence of underlying cardiac disease in patients with bacterial endocarditis observed from 1932 to 1943 and 1944 to 1960 showed no significant change other than an increase in the proportion of patients with congenital heart disease. The proportion of patients with rheumatic heart disease, no underlying heart disease, or heart disease of undetermined origin was essentially the same during the two periods (Table 1). In contrast, the proportion of patients with congenital heart disease showed a significant increase from 6% in the earlier series to 15% in the later series ($P<0.01$). This increase is probably primarily

related to new and accurate diagnostic methods such as angiocardiography and cardiac catheterization, and the prolongation of life by cardiac surgery.

TABLE 1. *Underlying heart disease*

| Type of heart disease | 1932 to 1943 173 Patients [a] | | 1944 to 1960 210 Patients [b] | | Chi-square test (P value) |
|---|---|---|---|---|---|
| | *Number* | % | *Number* | % | |
| Rheumatic | 73 | 42 | 82 | 39 | Not significant[c] |
| Congenital | 11 | 6 | 32 | 15 | Highly significant[d] |
| None | 21 | 12 | 16 | 8 | Not significant[c] |
| Undetermined origin[e] | 65 | 38 | 71 | 34 | Not significant[c] |
| Other types (syphilitic, arteriosclerotic, Libman-Sachs endocarditis) | 5 | 3 | 10 | 5 | Not significant[c] |

[a] In this group 2 patients had both rheumatic and congenital heart disease.

[b] Only 1 patient had both rheumatic and congenital heart disease.

[c] $P > 0.05$

[d] $P < 0.01$

[e] The patients in this group did not meet the criteria as defined in this paper for diagnosis of a specific cardiovascular disease. As explained in the text, it is probable that about one-half of these patients had rheumatic heart disease.

The group of patients with heart disease of undetermined origin, which constitutes about 35% of the entire series, deserves additional comment. Most of these patients were considered by their physicians to have rheumatic heart disease but evidence in support of this diagnosis was often scant. Diagnoses established at autopsy would suggest that about half of this group actually had rheumatic heart disease and that the rest had congenital heart disease, no heart disease, or other types of heart disease in approximately the same proportion as indicated in Table 1. It should also be noted that the incidence of clinically diagnosed rheumatic heart disease, derived by using criteria listed in this paper (i.e., a history of acute rheumatic fever or of murmur of mitral stenosis antedating onset of bacterial endocarditis), agreed quite well with the 46% incidence of rheumatic heart disease found at autopsy.

*Cure rates.* Only three patients were cured before 1944; one received penicillin and two received sulfonamides (Table 2). Of the entire series, 74% were cured from 1944 to 1951 with an 81% cure rate for endocarditis caused by viridans streptococci. The over-all cure rate was 83% from 1952 to 1960 with a 90% cure rate for endocarditis caused by viridans streptococci. Almost all of the patients with streptococcal disease who were cured received penicillin or a combination of penicillin and streptomycin. It is of interest to note (Table 2) that during the last nine years the cure rates for endocarditis caused by streptococci of the enterococcus and viridans groups

are identical. The improvement in cure rate for endocarditis caused by streptococci of the enterococcus group is probably due to use of a therapeutic regimen consisting of high dosages of aqueous penicillin and streptomycin (Koenig and Kaye, 1961).

TABLE 2. *Cure rates*

| Group | Total series | | Streptococci of the viridans group | | Streptococci of the enterococcus group | | Staphylococci | |
|---|---|---|---|---|---|---|---|---|
| | *Number* | % | *Number* | % | *Number* | % | *Number* | % |
| 1932 to 1943 | 3/173 | 2 | 1/111 | 1 | 0/4 | 0 | 0/13 | 0 |
| 1944 to 1951 | 86/117 | 74 | 69/85 | 81 | 7/11 | 64 | 3/8 | 38 |
| 1952 to 1960 | 77/93 | 83 | 47/52 | 90 | 9/10 | 90 | 7/13 | 54 |

*Incidence of etiological microorganisms.* Table 3 lists the incidence of etiological microorganisms for the two periods of the study. The proportion of cases of endocarditis caused by streptococci of the viridans group did not change appreciably from 1932 to 1943 (64%) to 1944 to 1960 (65%). However, the incidence of endocarditis caused by *D. pneumoniae* significantly decreased (8 to 1%), and the incidence of endocarditis caused by streptococci of the enterococcus group significantly increased (2 to 10%) (P values $< 0.01$). Staphylococci caused 7% of cases during 1932 to 1943 and 10% during 1944 to 1960. Therefore, the main change in incidence of etiological microorganisms was a decrease in proportion of *D. pneumoniae* with an equivalent increase in proportion of enterococci. The decreased incidence of *D. pneumoniae* endocarditis has been previously noted (Hunter and Paterson, 1956) and is probably related to widespread, early curative use of penicillin and other antimicrobials for pneumonia caused by these microorganisms. The increased incidence of enterococcal endocarditis also noted by others (Hunter and Paterson, 1956) is not easily explained. One factor may be that a longer life span subjects more males to urologic procedures, which predispose to enterococcal bacteremia and subsequent endocarditis (Robbins and Tompsett, 1951; Koenig and Kaye, 1961).

*Penicillin susceptibility of etiological microorganisms.* Since 1944 more than 90% of the microorganisms isolated from patients with bacterial endocarditis have been tested for susceptibility to penicillin by an an antibiotic dilution method in broth. Fig. 4 shows the minimal inhibitory concentration of penicillin for 79 strains of viridans streptococci, tested from 1944 to 1951, and 45 strains tested from 1952 to 1960. There is no significant difference in susceptibility of streptococci of the viridans group isolated during the two periods: 18% of the viridans streptococci from the earlier period

TABLE 3. *Etiological microorganisms*

| Organisms | 1932 to 1943 173 Patients[a] | | 1944 to 1960 210 Patients | | Chi-square test (P value) |
|---|---|---|---|---|---|
| | *Number* | % | *Number* | % | |
| Streptococci of the viridans group | 111 | 64 | 137 | 65 | Not significant[b] |
| Streptococci of the pyogenic group | 8 | 5 | 7 | 3 | Not significant[b] |
| Streptococci of the lactic group | 15 | 9 | 10 | 5 | Not significant[b] |
| Streptococci of the enterococcus group | 4 | 2 | 21 | 10 | Highly significant[c] |
| *Diplococcus pneumoniae* | 13 | 8 | 2 | 1 | Highly significant[c] |
| *Staphylococcus aureus* | 9 | 5 | 19 | 9 | Not significant[b] |
| *Staphylococcus epidermidis* | 4 | 2 | 2 | 1 | Not significant[b] |
| Others (*Bacteroides, Streptobacillus moniliformis, Paracolobactrum, Pseudomonas, Proteus mirabilis, Nocardia, Candida albicans, Vibrio fetus, Neisseria gonorrhoeae, Neisseria sicca,* microaerophilic streptococcus, anaerobic streptococcus, unidentified gram-negative coccobacillus) | 11 | 6 | 12 | 6 | Not significant[b] |

[a] In this group two etiologic microorganisms were isolated in two patients.
[b] $P > 0.05$
[c] $P < 0.01$

and 20% from the later period were resistant to 0.1 unit per ml of penicillin. Comparison of 37 strains from 1944 to 1946 with 15 strains from 1958 to 1960, the earliest and latest three-year periods of penicillin therapy, revealed no change in susceptibility to penicillin.

It has been reported that streptococci of the viridans group, isolated from the throats of children receiving small doses of penicillin to prevent recurrences of rheumatic fever, are significantly more resistant to penicillin than streptococci isolated from patients not receiving prophylaxis (Krumwiede, 1949). In view of this observation and the role of oral streptococci in the pathogenesis of bacterial endocarditis, an increasing incidence of endocarditis caused by penicillin-resistant streptococci might be anticipated. However,

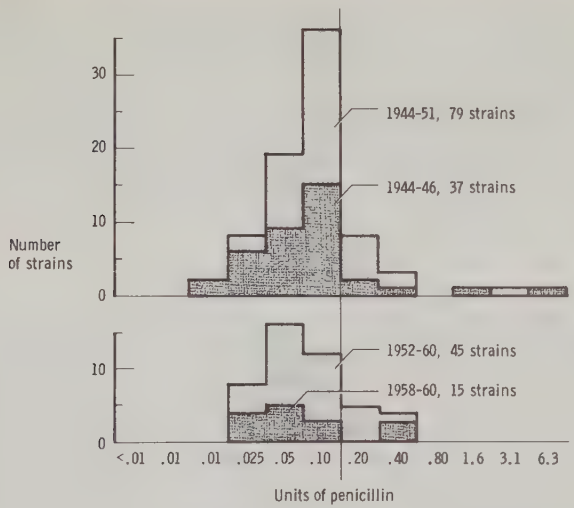


FIG. 4. *Penicillin susceptibility of 124 strains of streptococci of the viridans group isolated from blood specimens from 124 patients with bacterial endocarditis. The shaded areas represent strains from 1944 to 1946 and 1958 to 1960.*

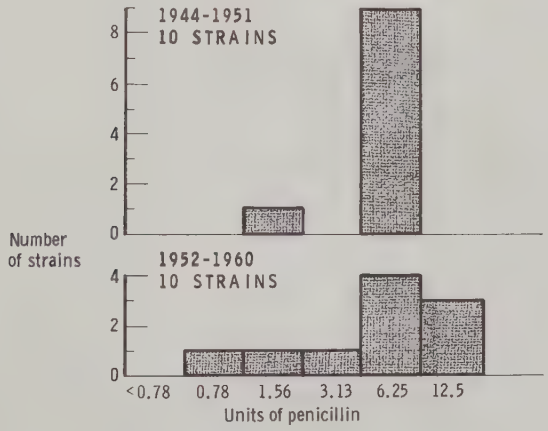


FIG. 5. *Penicillin susceptibility of 20 strains of streptococci of the enterococcus group isolated from blood specimens from 20 patients with bacterial endocarditis.*

the present study and the observations of Berntsen (1955) indicate that since the advent of antibiotic therapy there has been no significant change in penicillin susceptibility of streptococci of the viridans group isolated from patients with bacterial endocarditis.

In the present study there was no striking difference in penicillin susceptibility of 10 strains of enterococci isolated from 1944 to 1951 and 10 strains tested from 1952 to 1960 (Fig. 5). About one-half of the staphylococci tested from each period were resistant to 1 unit per ml of penicillin.

## Acknowledgments

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## Literature Cited

Anderson, H. J., and J. S. Staffurth. 1955. Subacute bacterial endocarditis in the elderly. Lancet 2:1055-1058.

Angrist, A., and J. Marquiss. 1954. The changing morphologic picture of endocarditis since the advent of chemotherapy and antibiotic agents. Am. J. Pathol. 30:39-63.

Berntsen, C. A., Jr. 1955. Unaltered penicillin susceptibility of streptococci. J. Am. Med. Assoc. 157:331-333.

Cummings, V., S. Furman, M. Dunst, and I. L. Rubin. 1960. Subacute bacterial endocarditis in the older age group. J. Am. Med. Assoc. 172:137-141.

Finland, M., W. F. Jones, Jr., and M. W. Barnes. 1959. Occurrence of serious bacterial infections since introduction of antibacterial agents. J. Am. Med. Assoc. 170:2188-2197.

Friedberg, C. K., H. M. Goldman, and L. E. Field. 1961. Study of bacterial endocarditis. A. M. A. Arch. Internal Med. 107:6-15.

Hartman, T. L., and W. K. Myers. 1959. Occurrence of bacterial endocarditis in older individuals. Geriatrics 14:374-380.

Hunter, T. H., and P. Y. Paterson. 1956. Bacterial endocarditis. DM:(Disease-a-Month) p. 1-48, November.

Koenig, M. G., and D. Kaye. 1961. Enterococcal endocarditis: report of nineteen cases with long-term follow-up data. New Engl. J. Med. 264:257-264.

Krumwiede, E. 1949. Penicillin resistance of nonhemolytic streptococci from rheumatic children receiving prophylactic penicillin. Pediatrics 4:634-642.

Littmann, D., and R. S. Schaaf. 1950. Therapeutic experiences with subacute bacterial endocarditis. New Engl. J. Med. 243:248-252.

Robbins, W. C., and R. Tompsett. 1951. Treatment of enterococcal endocarditis and bacteremia: results of combined therapy with penicillin and streptomycin. Am. J. Med. 10:278-299.

Rogers, D. E. 1959. The changing pattern of life-threatening microbial disease. New Engl. J. Med. 261:677-683.

# SEASONAL STAPHYLOCOCCAL CARRIER RATES IN A POPULATION OF "NORMAL" FAMILIES

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Although many aspects of the problem of staphylococcal infections have been investigated extensively in the hospital environment, relatively little is known of the nonhospital, staphylococcal carrier state. This is rather surprising because, even though infections with coagulase-positive staphylococci (CPS) present a very real problem, the largest proportion still is acquired in the community. With this in mind, the present study was undertaken to gain some knowledge of the staphylococcal carrier status of the ambulant population. It was further designed so as to provide information on the relative importance of such factors as age, sex, and season.

## Materials and Methods

As many as 130 families (some dropped out from time to time), who reside in a low-cost housing project adjacent to the Upstate Medical Center, made up the population under observation. The families are under continuing study for respiratory diseases. The staphylococcal surveys were made by obtaining nasal cultures from the total population four times a year, once in each of the seasons. The swabs were planted on Trypticase soy agar containing 7.5% NaCl. Typical colonies were picked and tested for coagulase production by the tube method. Those that produced coagulase were studied further for their bacteriophage types and their susceptibility to various antibiotics (disc method).

The population is relatively young and contains a high proportion of preschool and school age children. Since only families with school-age children were studied, this high proportion is not surprising. There were few persons over 35 or under 1 year of age. This age distribution has remained fairly constant throughout the observation period.

## Results

Of the 3,567 cultures that were obtained during the 11 consecutive seasonal surveys, 1,219 (33%) contained CPS. The most frequent carriers were school-age children. Preschool children were carriers less often and older individuals even less so. There was no significant sex difference, although males tended to carry CPS slightly more often than females.

Although 26 to 35% of the study group harbored CPS during winter, spring, and fall, 44% were carriers during the summer surveys. The seasonal difference in age-specific carrier rate is even more striking when only the data from the 5 to 14 age group is considered. The difference in carrier rates between the winter and summer sampling periods is marked, 37% compared to 57%.

The square footage of each apartment is known, so the degree of crowding can be readily determined from the number of individuals occupying a given apartment. Thus, families are classified as "crowded" or "uncrowded" for comparison with one another. In view of the seasonal variations in carrier rates, one of the questions that arose was the effect of crowding on the carrier rates for CPS. There was little, if any, difference between the "crowded" and "uncrowded" families.

We next compared individuals sleeping in bedrooms where there was less than 30 sq ft per individual with those occupying rooms with more than 60 sq ft per individual. Again, no differences in carrier rates for CPS could be ascertained.

Several investigators have suggested that there are three types of "carriers": persistent, intermittent, and noncarriers. Except for Roodyn (1960), most such investigations have been conducted for less than 1 year. In the present study, similar kinds of carriers were noted. Among 424 individuals who were studied for 1 to 2 years, 85 (20%) never had a positive culture for CPS. An additional 100 individuals were found to have CPS on only one occasion. Twelve (3%) persons, however, were positive in each survey, and an additional 35 (8%) were negative only once. The remaining 198 (45%) could be considered to be intermittent carriers. The persistent carriers always carried staphylococci of the same phage type; the intermittent carriers were inconsistent as to type.

Families can be classified in a similar fashion. Some families rarely contained members who were carrying CPS; in others, there were always several members who harbored CPS, usually of the same phage group and type.

The CPS have been examined for their susceptibility to various antibiotics. Among 903 strains tested with a 2-unit penicillin disc, 68% were inhibited by this concentration. A 10-unit disc inhibited 779 strains (91%) of CPS. Only 35% of the strains were susceptible to a 2-$\mu$g disc of streptomycin, but 97% were sensitive to the 10-$\mu$g

disc. Almost 100% of the strains were inhibited by 5-$\mu$g discs of tetracycline and chloramphenicol or a 2-$\mu$g disc of erythromycin. Consequently, it can be concluded that most of the CPS isolated from this nonhospital community were sensitive to the commonly employed antibiotics. There were no significant differences in antibiotic sensitivity of CPS isolated in different seasons of the year or from different age groups.

Approximately 25% of the staphylococci have been phage typed to date. Staphylococci sensitive to phages of groups I, II, and III predominate. There was no age, sex, or seasonal predominance noted for any one phage type or group. Some 18% of the staphylococci were not identifiable with any of the bacteriophages.

## Discussion

The present study indicates that nonhospital carriage of CPS varies with age and season. Several investigators have similarly noted increased carrier rates in the school age child. Of interest is the demonstration of the persistence of a particular strain of staphylococci in some individuals and the apparent inability of other individuals to acquire CPS.

The seasonal variation in staphylococcal carrier rates apparently has not been noted previously. What relation this has to outbreaks of staphylococcal disease cannot be determined at present. However, several investigators have noted that the peak incidence of staphylococcal infections is during the summer months. This seasonal factor probably should be considered in any proposed system for controlling staphylococcal infections.

## Acknowledgments

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## Literature Cited

Roodyn, L. 1960. Recurrent staphylococcal infections and duration of the carrier state. J. Hyg. 58:11-19.

# OCCURRENCE OF *ACANTHAMOEBA* IN TISSUE CULTURES INOCULATED WITH HUMAN PHARYNGEAL SWABS

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Since the initial paper by Jahnes, Fullmer, and Li (1957), there have been two other reports (Culbertson et al., 1959; Chi, Vogel, and Shelokov, 1959) which describe the finding of amebae of the genus *Acanthamoeba (Hartmannella)* in monkey-kidney tissue cultures. Although Chi et al. encountered these organisms in a tube inoculated with a throat swab from a child of unstated age, they were inclined to believe that the source of the amebae was the monkey-kidney tissue culture rather than the patient. Each group of authors believed that they had encountered a new "virus," only to find, subsequently, that they were passing a free-living ameba considered to be a normal soil or water inhabitant. Our experience was the same. Culbertson (1961) later demonstrated that these organisms may be pathogenic for monkeys, mice, and rabbits and that "antibodies" may be detected by complement fixation (CF).

In the present study, only 1 of 19 isolations of these organisms was made in monkey-kidney cultures. The other 18 were observed in either HeLa or Hep-2 cell cultures, so that we are not concerned with the question of a spontaneous infection of monkey-kidney cells.

## Materials and Methods

Our isolations have been made since the fall of 1958 in the course of a study of respiratory disease in a population of normal families who reside in a low-cost housing development adjacent to the Medical College in Syracuse. More than 10,000 pharyngeal swabs have been inoculated into HeLa and Hep-2 tissue culture tubes. Monkey-kidney cells were used occasionally in the early part of the study. From 19 swabs (18 individuals) free-living amebae have been identified as members of the genus *Hartmannella*, of which three species are represented: *rhysodes*, *agricola*, and *glebae*.

## Results and Discussion

The amebae have been found in both sexes and in all seasons. Thirteen subjects were under 2 years of age and only one was more than 9 years old. The latter was a 28-year-old female, who was of special interest because two isolations of *H. rhysodes* were made from throat swabs obtained 1 month apart. Type 5 adenovirus was subsequently isolated from one of these. Several serum samples from her were fortuitously available for serological study.

The organism can be propagated with ease in different cell lines, such as HeLa, Hep-2, monkey and calf kidney cells. Both trophozoites and cysts may be seen. Trophozoites in Hep-2 cells produce no specific cytopathic change nor is intracellular growth observed. Toxin formation does not seem to occur. When numbers of trophozoites accumulate, intensive degeneration of the tissue-culture cells may be seen. It appears as though the fine particles from dead tissue cells are utilized by the vegetative forms of the amebae. Multiplication occurs readily, however, in the absence of cells and bacteria. After a number of trials, we found a medium consisting of 20% heated calf serum and 10% Trypticase soy broth in Hank's balanced salt solution with 0.5% gelatin at pH 7 to produce excellent growth of trophozoites. In this medium the trophozoite population doubles every 48 hr, for at least 144 hr.

In the synthetic medium, finger-like, protoplasmic projections from the trophozoites and food vacuoles may be noted. The cysts are variable both as to size and shape. They are double-walled, irregular in outline, and have a wrinkled appearance. There are pores in the cyst wall. Encystment is not necessarily a phenomenon of aging. Unfavorable pH, freezing, shaking, or other physical agitation result in prompt encystment. The cysts may be removed by filtration or simple centrifugation.

In trophozoites which have been stained with hematoxylin-eosin, a single nucleus with a large karyosome, a single contractile, and many food vacuoles may be seen. The finger-like pseudopodia are not present in the stained specimens.

Culbertson (1961) reported that the strain of amebae which he isolated from an uninoculated monkey-kidney tissue culture is pathogenic for monkeys, mice, and rabbits. We have not attempted to duplicate these experiments, but we have injected quite heavy doses of trophozoites into the bloodstreams of rabbits and guinea pigs without inducing apparent illness or death.

We have tried to immunize a few rabbits with both cysts and trophozoites of *H. rhysodes* cultivated in different cell lines or in the liquid medium. The dosage of cysts was measured in terms of the infectious dose after titration in tissue culture. The concentration of trophozoites was determined by actual count. CF tests were performed with an antigen made from formalinized *H. rhysodes*.

Neutralization tests were performed with dilutions of serum and 100 $ID_{50}$ of cysts in 0.2 ml or against 3,000 trophozoites in 0.1 ml. The end point was the highest dilution of serum which inhibited the growth of amebae.

All normal rabbit sera was found to react in the CF test. Their titers were unaffected by immunization. On the other hand, neutralizing antibody was not detected in normal rabbit sera. It appeared after a series of injections, but attained only a low level. These antigens are far from satisfactory and studies are in progress to devise better ones.

Serological tests with human sera are limited and somewhat confusing. A group of 100 sera selected at random from specimens submitted for antistreptolysin-O determination from patients aged 1 to 22 years was examined in the CF test. They were collected during the period January to August 1961. Two-thirds were negative or positive only in the undiluted tube. The titers then varied from four positive at 1:32, to one at 1:128. There is no positive correlation between age and titer. The meaning of these reactions is obscure at present.

Another set of data, obtained from the 28-year-old female from whom there were two isolations of amebae, demonstrate results similar to those noted in the immunized rabbits. The CF titers are of no help, but the neutralization tests suggest conversion from a negative to a positive level.

Although free-living amebae may occur spontaneously in monkey-kidney tissue cultures, it is apparent that they may, also, be isolated in other cell lines from human throat swabs. Whether the greater incidence in young children and infants is a reflection of a greater tendency to get dirt into the mouth can only be surmised. In tissue culture, these protozoa can be confused with the true viruses. That they may produce disease in humans or animals is possible, but remains the subject of current investigations.

## Acknowledgments

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## Literature Cited

Chi, L., J. E. Vogel, and A. Shelokov. 1959. Selective phagocytosis of nucleated erythrocytes by cytotoxic amoebae in cell culture. Science. 130:1763.

Culbertson, C. G. 1961. Pathogenic *Acanthamoeba* (Hartmannella). Am. J. Clin. Pathol. 35:195-202.

Culbertson, C. G., J. W. Smith, H. K., Cohen, and J. R. Minner. 1959. Experimental infection of mice and monkeys by *Acanthamoeba*. Am. J. Pathol. **35**:185-197.

Jahnes, W. G., H. M. Fullmer, and C. P. Li. 1957. Free living amoebae as contaminants in monkey kidney tissue culture. Proc. Soc. Exptl. Biol. Med. **96**:484-488.

# ESTABLISHMENT AND TREATMENT OF EXPERIMENTAL *ESCHERICHIA COLI* PYELONEPHRITIS

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To insure local and persistent experimental renal infections with gram-negative bacteria, a productive approach has been the employment of an ever-increasing variety of methods for altering resistance. Thus, kidney massage (Braude, Shapiro, and Siemienski, 1955; Shapiro, Braude, and Siemienski, 1956), ureteral ligation (Guze and Beeson, 1956), and various foreign-body implantations (Kass, 1960; Miller et al., 1956) have been employed successfully to increase susceptibility. In addition to the intravascular route of bacterial dissemination, recent clinical and experimental evidence implicates bacteriuria as a significant factor in the induction of pyelonephritis via the ureteral lumen (Kass, 1960; Kleeman, Hewitt, and Guze, 1960). Indeed, either route of inoculation has been capable of initiating urinary tract infections. For the most part, these studies have provided "model" systems, not only for comparative histologic investigations but also for the evaluation of host-parasite-drug interactions. It is generally recognized, moreover, that uncomplicated, acute *Escherichia coli* pyelonephritis is amenable to therapeutic agents, whereas complicated infections involving anatomic dearrangement are relatively resistant to treatment.

To resolve and integrate certain aspects of *E. coli* pyelonephritis, it was felt that two issues had to be clarified further: first, the influence of therapeutic agents possessing urinary- and plasma-inhibitory concentrations in uncomplicated and complicated infections; and second, provision of histologic observations on the course of these experimental infections. The present work is concerned with the first of these two issues and shows that the dose regimen of either nitrofurantoin or chloramphenicol and the initial time of administration after infection affect the degree to which these agents were effective. The second issue is the subject of the following report, in which it is shown that the hematogenous and retrograde pathways of bacterial dissemination to normal kidneys initiate and develop similar pathologic changes in contrast to injured kidneys.

## Materials and Methods

*Culture.* *E. coli*, strain ES-20, was originally isolated from a case of calf scours. The culture was grown on meat-extract agar for 16 hr at 37 C and suspended in Ringer's solution. The optical density was determined spectrophotometrically and correlated to a reference optical density vs. viable cell graph.

*Animals.* Male, Wistar rats, weighing 350 g, and male, CFW mice, weighing 20 g were used.

*Assay procedure.* At various times after infection, the kidneys were removed; one-half of each kidney was preserved for histopathologic examination and the corresponding half was mascerated in Ringer's solution and plated on eosin-methylene blue agar for quantitative viable cell counts.

*Therapeutic agents.* Nitrofurantoin and chloramphenicol were orally administered in sodium carboxymethylcellulose.

## Results

*Bacteriological activity.* Serial twofold dilutions of nitrofurantoin (NF) and chloramphenicol (CM) were made in broth and inoculated with $1 \times 10^4$ *E. coli* cells. The minimum inhibitory concentrations of these agents upon incubation at 37 C for 24 hr were comparable at 10 $\mu$g/ml.

TABLE 1. *Antibacterial levels in rat urine and serum following oral administration of nitrofurantoin and chloramphenicol*

| Drug | Dose | Inhibitory dilution against *F. coli* | | |
|---|---|---|---|---|
| | | Urine | Serum | |
| | *mg/kg* | | *1 hr* | *2 hr* |
| NF | 10 | 1:20 | <1:2 | <1:2 |
| | 25 | 1:80 | <1:2 | <1:2 |
| CM | 10 | 1:5 | 1:2 | <1:2 |
| | 25 | 1:20 | 1:8 | 1:2 |
| | 50 | 1:160 | 1:16 | 1:4 |

Pooled rat urines were collected over a 4-hr period following the oral administration of 10 and 25 mg/kg of NF and CM. In addition, serum samples were obtained at 1 and 2 hr. The antibacterial levels of the urine and serum samples, given in Table 1, show that NF activity is essentially limited to urine concentration. A dose of 10 mg/kg which inhibited *E. coli* at 1:20 urine dilution was comparable to CM at a dose of 25 mg/kg. On the other hand, CM produced inhibitory serum levels of at least 2-hr duration following dosages of 25 and 50 mg/kg.

*Renal infections in rats.* In orientation experiments to determine the dose of *E. coli* cells required for consistent infections, groups of 30 rats were injected with varying cell concentrations directly into the bladder (IB) via an abdominal incision. Alternatively, the intracardiac route (IC) was also used. At 2, 6, 10, 20, 30, and 40 days, 5 rats from each group were autopsied and examined for the presence of viable cells in the kidneys, urines, livers, and spleens.

The results showed that the IB route with $5 \times 10^9$ cells produced 90 to 100% kidney involvement 2 days post infection. This incidence declined at the tenth day to a constant level of approximately 60% for periods up to 40 days. The bacterial counts in the kidneys and urines were in the range of $1 \times 10^5$ to $1 \times 10^6$ cells and were limited to the urinary tract. The inoculation of less than $1 \times 10^9$ cells was below the minimum infectious dose. On the other hand, the maximum nonlethal IC inoculation was $5 \times 10^8$ cells and produced a comparable pattern, although the initial infection was approximately 70%. Viable cells were not recovered from the livers and spleens after 6 days but were present in the urines at 2 days. Rats receiving less than the maximum IC challenge were sterile after 6 days.

*Therapeutic activity in rat infections.* The experimental design of these experiments was essentially to infect groups of rats via the IB and IC routes and to administer one or more dose regimens of nitrofurantoin and chloramphenicol. In one series, treatment was started 6 hr after infection for a 2-day period; in the other series, treatment was started at 6 and 48 hr after infection for periods of 8 to 10 days. The criterion for cure was the absence of viable cells upon culture of kidney homogenates. Plate count enumerations were in the range of $1 \times 10^5$ cells, not only in the controls, but also in kidneys which failed to respond to treatment.

TABLE 2. *Effect of nitrofurantoin and chloramphenicol on rat E. coli pyelonephritis at 2 days post infection*

| Route infected | Drug | Dose | No. of doses* | No. of rats | Percent sterile | |
|---|---|---|---|---|---|---|
| | | | | | Rats | Kidneys |
| | | *mg/kg* | | | | |
| IB | NF | 25 | 1 | 8 | 0 | 25 |
| | NF | 2.5 | 4 | 15 | 46.6 | 46.6 |
| | NF | 10 | 4 | 20 | 50 | 65 |
| | NF | 25 | 4 | 20 | 65 | 77.5 |
| | CM | 25 | 1 | 8 | 12.5 | 37.5 |
| | CM | 10 | 4 | 20 | 20 | 47.5 |
| | CM | 25 | 4 | 20 | 40 | 57.5 |
| | Control | - | - | 20 | 5 | 15 |
| IC | NF | 10 | 4 | 15 | 75 | 83.3 |
| | CM | 10 | 4 | 15 | 60 | 73.3 |
| | CM | 25 | 4 | 15 | 96.6 | 90 |
| | Control | - | - | 15 | 20 | 36.6 |

*First dose administered 6 hr after infection.

In Table 2, the results of different dose regimens over 2 days are given and show that the dissemination of bacteria via the hematogenous and retrograde routes was amenable to nitrofurantoin or chloramphenicol therapy. These agents were highly effective at 10 mg/kg against the IC route, producing 75 to 85% cures. On the other hand, ascending infections responded more readily to 2.5 and 10 mg/kg of NF than to 10 and 25 mg/kg of CM. This dose-response relationship indicated that from 45 to 65% clearance was obtained with 4 doses of NF at 2.5 and 10 mg/kg or after 4 doses of CM at 10 and 25 mg/kg.

The effect of continued therapy is presented in Table 3 and shows further that these dose relationships increased the percentage of sterile kidneys to almost 100% in 10 days. When therapy was started 2 days after infection via the IC and IB routes, 10 mg/kg of either drug had essentially the same efficacy of approximately 70 to 80% clearance. However, earlier treatment was more effective.

TABLE 3. *Effect of nitrofurantoin and chloramphenicol on rat E. coli pyelonephritis at 10 days post infection*

| Route infected | Drug | Initial dose | Dose | No. of doses | No. of rats | Percent sterile | |
|---|---|---|---|---|---|---|---|
| | | | | | | Rats | Kidneys |
| | | *hr* | *mg/kg* | | | | |
| IB | NF | 6 | 10 | 10 | 15 | 80 | 90 |
| | NF | 6 | 10 | 20 | 15 | 93.3 | 96.6 |
| | CM | 6 | 25 | 10 | 15 | 53.3 | 70 |
| | CM | 6 | 25 | 20 | 15 | 100 | 100 |
| | NF | 48 | 10 | 16 | 18 | 72.2 | 75 |
| | CM | 48 | 10 | 16 | 18 | 72.2 | 77.7 |
| | Control | - | - | - | 24 | 25 | 42 |
| IC | NF | 48 | 10 | 16 | 12 | 50 | 70.9 |
| | CM | 48 | 10 | 16 | 12 | 58.3 | 79.2 |
| | Control | - | - | - | 12 | 25 | 37.5 |

*Renal infections in mice.* To produce renal stress to enhance the infectious process, preliminary experiments were made using intravenous inoculations of rabbit erythrocytes and *E. coli* cells. It was determined that 0.2 ml of a 20% erythrocyte suspension containing $4 \times 10^8$ bacteria caused a transient hematuria and a 75% incidence of kidney infection. Visible abscesses were observed by the sixth day and persisted for over 40 days. Bacteriuria was present at the second day, whereas the livers and spleens were sterile at 6 days. Viable bacterial counts in the range of $5 \times 10^6$ cells were obtained from kidneys and urines. It may be well to point out that the inoculation of *E. coli* without erythrocytes gave inconsistent and transient infections.

Additional studies were made wherein hydronephrosis was produced by the ligation of the right ureters of anesthesized mice via

an abdominal incision. Three days later, $5 \times 10^7$ cells were inoculated intravenously. This procedure produced 100% infections in the ligated kidneys with viable counts exceeding $1 \times 10^8$ cells and persisting for over 40 days. These infections were limited to the ligated kidneys.

TABLE 4. *Effect of immediate and delayed treatment with nitrofurantoin and chloramphenicol on mouse E. coli pyelonephritis*

| Drug | Initial dose* | Dose | No. of doses | Percent sterile† | |
|---|---|---|---|---|---|
| | | | | Mice | Kidneys |
| | *hr* | *mg/kg* | | | |
| NF | 0 | 20 | 8 | 35 | 45 |
| | 0 | 20 | 16 | 70 | 72.5 |
| | 0 | 50 | 8 | 80 | 85 |
| | 0 | 50 | 16 | 90 | 92.5 |
| | 48 | 50 | 8 | 55 | 70 |
| | 48 | 50 | 16 | 70 | 75 |
| CM | 0 | 20 | 8 | 35 | 57.5 |
| | 0 | 20 | 16 | 85 | 90 |
| | 0 | 50 | 8 | 100 | 100 |
| | 48 | 20 | 16 | 45 | 55 |
| | 48 | 50 | 8 | 65 | 72.5 |
| | 48 | 50 | 16 | 80 | 90 |
| Control | – | – | – | 20 | 27.5 |

*Drugs administered twice daily.
†Twenty mice per group.

TABLE 5. *Effect of nitrofurantoin and chloramphenicol on mouse E. coli pyelonephritis with obstructed ureters*

| Drug | Initial dose* | Dose | No. of doses | No. of mice† | Percent sterile |
|---|---|---|---|---|---|
| | *hr* | *mg/kg* | | | |
| NF | 0 | 50 | 8 | 5/15 | 33.3 |
| | 0 | 50 | 16 | 9/12 | 75 |
| | 48 | 50 | 8 | 0/10 | 0 |
| | 48 | 50 | 16 | 3/12 | 25 |
| CM | 0 | 50 | 8 | 6/10 | 60 |
| | 0 | 50 | 16 | 11/12 | 91.7 |
| | 48 | 50 | 8 | 2/10 | 20 |
| | 48 | 50 | 16 | 5/12 | 41.6 |
| Control | – | – | – | 0/15 | 0 |

*Drugs administered twice daily.
†Sterile mice over total no. used.

*Therapeutic activity in complicated mouse infections.* The results of chemotherapy against *E. coli* infections accelerated by erythrocytes are given in Table 4. When treatment of 20 mg/kg was started at the time of infection, the percentages of cures with NF and CM

increased in relation to the number of doses administered. Thus, this increase from 8 to 16 doses was approximately 30%. At 50 mg/kg, CM cleared 100% of the mice where 85% were cleared with NF. The data also indicate that 8 doses of 50 mg/kg were comparable to 16 doses of 20 mg/kg. Dose regimens with both drugs started after a 48-hr delay were not as effective as immediate treatment but, nevertheless, eliminated over 70% of infected kidneys at 50 mg/kg. On the other hand, this dose level was considerably less effective against infected hydronephritic kidneys (Table 5) although prolonged treatment increased the percentage of cures from 20 to 25%.

## Discussion

This investigation had, as its primary objective, the determination of nitrofurantoin and chloramphenicol activity in uncomplicated and complicated *E. coli* pyelonephritis. It was readily shown that the kidneys of normal rats were accessible to infections by the hematogenous route and by ascending via the ureteral lumen. McCabe and Jackson (1960) have shown that the retrograde route produces an early phase of bacterial sequestration before active proliferation. The results reported here, however, demonstrate that an immediate and progressive infection was established with the use of large cell inocula. Histopathologic studies (Van Ryzin et al., 1962) also confirmed this observation of a rapid tissue infection. These uncomplicated infections were amenable to treatment, not only with nitrofurantoin which is active through filtration and urine concentration, but also with chloramphenicol which is primarily inhibitory in plasma. It is appreciated, however, that high doses of chloramphenicol produce, in addition, urine inhibitory levels. Indeed, urine levels after a dose of 10 mg/kg of nitrofurantoin agreed to those obtained with 25 mg/kg of chloramphenicol. This relationship also resulted in a comparable percentage of cures at these dosages. Moreover, increased dose regimens with both drugs accelerated the elimination of viable cells from infected kidneys. Previous studies have shown that the requirement for high levels of antibacterial activity in urine was a more useful predictor of efficacy and a requirement for cure (McCabe, Jackson, and Gribble, 1959).

The establishment of complicated mouse infections possessing high bacterial counts and abscesses required up to 100 mg/kg a day of these drugs to obtain almost complete elimination of infection. The response in hydronephritic kidneys was greatly reduced although progressive clearance was obtained with prolonged treatment regimens. In the acute infections produced by complete ureteral blockage, the drugs were, nevertheless, capable of penetrating to the site of infection.

## Literature Cited

Braude, A. I., A. P. Shapiro, and J. Siemienski. 1955. Hematogenous pyelonephritis in rats. I. Its pathogenesis when produced by a simple new method. J. Clin. Invest. **34**:1489-1497.

Guze, L. B., and P. B. Beeson. 1956. Experimental pyelonephritis. I. Effect of ureteral ligation on the course of bacterial infection in the kidney. J. Exptl. Med. **104**:803-815.

Kass, E. H. 1960. Bacteriuria and pyelonephritis of pregnancy. A. M. A. Arch Internal Med. **105**:194-198.

Kleeman, C. R., W. L. Hewitt, and L. B. Guze. 1960. Pyelonephritis. Medicine **39**:3-116.

McCabe, W. R., G. G. Jackson, and H. G. Gribble. 1959. Treatment of chronic pyelonephritis. A. M. A. Arch. Internal Med. **104**:710-719.

McCabe, W. R., and G. G. Jackson. 1960. The natural course of retrograde infections of the urinary tract of rats with different serotypes of *Escherichia coli* or Enterococcus, p. 39-52. *In* E. L. Quinn and E. H. Kass, [ed.], Biology of pyelonephritis. Little, Brown & Co., Boston.

Miller, G. H., W. H. Chapman, L. Seibutis, and C. W. Vermeulen. 1956. Furadantin treatment of experimental urinary tract infections and its influence on stone growth. XI. Experimental urolithiasis. J. Urol. **76**:42-46.

Shapiro, A. P., A. I. Braude, and J. Siemienski. 1956. Hematogenous pyleonephritis in rats. II. Production of chronic pyelonephritis by *Escherichia coli*. Proc. Soc. Exptl. Biol. Med. **91**:18-24.

Van Ryzin, R. J., B. Pitsch, T. Hebert, and W. F. Carey. 1962. Histological observations on experimental *Escherichia coli* pyelonephritis. Antimicrobial Agents and Chemotherapy - 1961, p. 61-66.

# HISTOLOGICAL OBSERVATIONS ON EXPERIMENTAL *ESCHERICHIA COLI* PYELONEPHRITIS

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This paper is a description and interpretation of histological changes observed in pyelonephritis, experimentally produced with *Escherichia coli*. The study was conducted to provide a basis for comparison of experimental pyelonephritis with pyelonephritis in man.

## Methods

Kidneys studied were from animals selected from experiments previously reported (Pitsch, Hebert, and Carey, 1962). One kidney from each rat was examined microscopically. Standard histological techniques were used to prepare the kidneys. Serial sections were not examined, but rather, single transverse and longitudinal sections of each kidney were studied. Microscopic findings were recorded without knowledge of the experimental treatment which the animal being examined received. Histological grading was done by one pathologist (R.J.V.R.) using an arbitrary estimate of the severity of pathological changes present.

## Results

*Development of lesions in rat kidneys.* Intrabladder (IB) inoculation with *E. coli* caused hyperplasia of renal pelvic epithelium, and leukocytic infiltration into the pelvic epithelium and the connective tissue beneath it (Fig. 1B and C). These changes were most often seen at the fornix of the pelvic space and along the parietal pelvic epithelium, but were not confined to these sites (Fig. 1A).

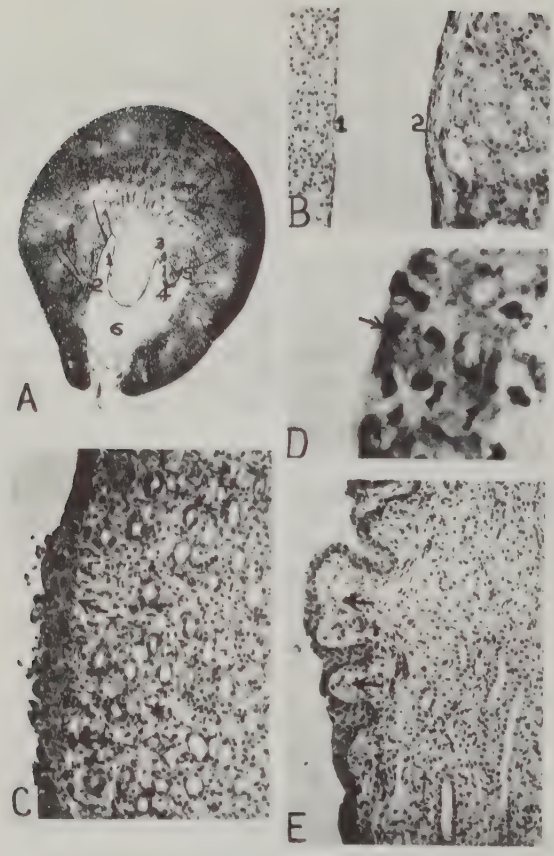


FIG. 1. A. *Pyelitis in a rat kidney 2 days after 1B inoculation with Escherichia coli. Macrophotograph: H & E × 7. Papillary pelvic epithelial lining* (1) *and parietal lining* (2) *are thickened. Thickened epithelium is particularly prominent at the fornix* (5) *of the pelvic space and covers a projection of medullary tissue* (4) *into the pelvis between the fornix and the apex* (3) *of the pelvic space* (6). *This kidney had a positive bacterial culture.* B. *Normal pelvic epithelium in an uninfected rat kidney. Photomicrograph: H & E × 185. Papillary pelvic epithelial lining* (1) *and parietal lining* (2) *are both a single cell layer in thickness. Compare with following photomicrographs.* C. *Higher magnification of papillary pelvic epithelium in the kidney shown in 1A. Photomicrograph: H & E × 185. The epithelium is seen to be 2 to 3 cell layers thick and leukocytes are in the epithelium and just beneath it (arrow).* D. *Colony of bacteria in pelvic epithelium in same kidney shown in 1A and 1C. Photomicrograph: Gridley reticulum stain × 1150. The bacteria (arrow), which are presumably E. coli, are short rods in the center of a semicircle of 5 leukocytes which are slightly out of focus.* E. *Chronic pyelitis in a rat kidney 20 days after 1C inoculation with E. coli. Photomicrograph: H&E × 185. The thickened epithelium is thrown into folds due to proliferation of subepithelial connective tissue (arrows), which still contains a few leukocytes. This kidney was bacteriologically sterile and from a nonmedicated animal.*

Infiltrating leukocytes, 2 days after inoculation, were mostly polymorphonuclear granulocytes, and the thickened pelvic lining was roughened in spots (Fig. 1C). After 18 to 20 days most of the leukocytes were monocytes and lymphocytes, and the pelvic lining was thrown up into folds due to proliferation of subepithelial connective tissue (Fig. 1E). By means of silver staining (Armed Forces Institute of Pathology, 1957), short rods, presumably *E. coli*, were demonstrated in the center of intraepithelial groups of leukocytes (Fig. 1D).

Renal pelvic inflammation (pyelitis) as described above was also seen in rats that were given intracardiac (IC) inoculation with *E. coli*.

After 2 days kidneys from IB-inoculated rats did not have inflammatory changes between tubules of the medulla and cortex (i.e., interstitial nephritis). After 10, 18, and 30 days, pyelitis was sometimes accompanied by interstitual nephritis; thus, pyelonephritis was then present. Some cortico-medullary lesions were so extensive they were grossly visible as gray triangular areas with their bases at the subcapsular cortex and apices at bases of renal papillae (Fig. 2A). Intertubular, cellular exudate contained mostly monocytes and lymphocytes, but 10-day specimens had many polymorphonuclear leukocytes also. Renal tubules involved in the cellular infiltration were often dilated and contained leukocytes or hyaline casts in their lumina (Fig. 2B and C). Early connective tissue replacement of destroyed cortical tubules was evident in larger lesions at 10 days, and fibrosis of parietal Bowman's capsules of renal corpuscles was seen after 30 days (Fig. 2D).

IC inoculation caused similar interstitial nephritis as did IB. In a few IC cases, this lesion was present as early as 2 days and was sometimes unaccompanied by pyelitis.

*Incidence of kidney lesions in rats and correlation with bacterial cultures.* Table 1 shows that incidence of pyelitis was high following IB inoculation, but only some kidneys had extension of inflammation into the kidney parenchyma resulting in pyelonephritis. Incidence of pyelitis was less in IC-inoculated rats. However, per cent of incidence

TABLE 1. *Incidence of histological renal lesions in rats inoculated with Escherichia coli*

| Route of inoculation | Days after inoculation | Number of kidneys examined | Kidneys with pyelitis | | Kidneys with pyelonephritis | | Kidneys with positive bacteriological culture | |
|---|---|---|---|---|---|---|---|---|
| | | | *Number* | % | *Number* | % | *Number* | % |
| Urinary bladder | 2 | 9 | 8 | 88 | 0 | 0 | 7 | 78 |
| | 10 | 17 | 14 | 82 | 2 | 12 | 10 | 59 |
| | 18 | 6 | 6 | 100 | 2 | 33 | 3 | 50 |
| | 30 | 12 | 7 | 58 | 2 | 16 | 4 | 33 |
| Heart blood | 2 | 4 | 1 | 25 | 1 | 25 | 2 | 50 |
| | 18 | 11 | 7 | 64 | 3 | 27 | 3 | 27 |

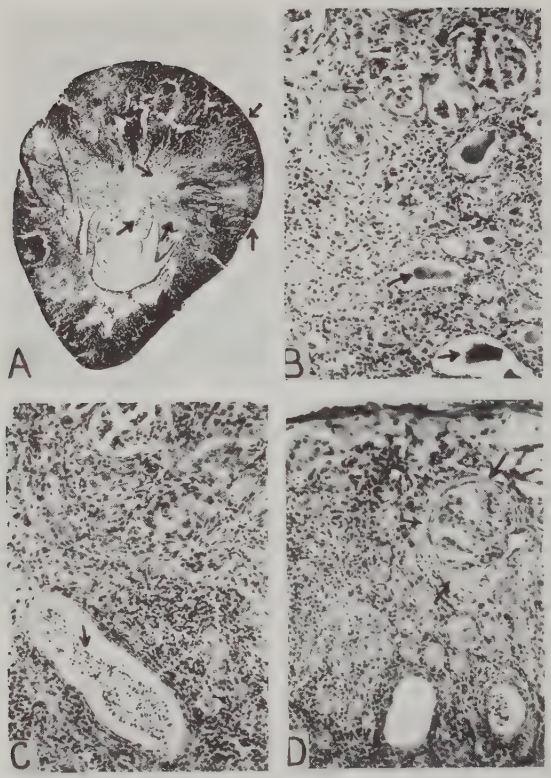


FIG. 2. A. *Pyelonephritis in a rat kidney 10 days after IB inoculation with Escherichia coli. Macrophotograph: H&E × 7. A large wedge-shaped lesion (arrows) extends from the base of the papilla to the subcapsular cortex. Dilated renal tubules are visible within the lesion. This kidney had a positive bacterial culture.* B. *Interstitial nephritis in the cortex of a rat kidney 18 days after IB inoculation with E. coli. Photomicrograph: H&E × 185. There is marked intertubular infiltration by leukocytes and dilated tubules containing colloid casts (arrows). This kidney had a positive bacterial culture.* C. *Interstitial nephritis in the cortex of a rat kidney 18 days after IC inoculation with E. coli. Photomicrograph: H&E × 185. This is intertubular leukocytic infiltration, similar to 18 day IB kidney in Fig. 2B, and a dilated tubule containing a cast made up of leukocytes. This kidney also had pyelitis but was bacteriologically sterile and from a nonmedicated control animal.* D. *Cortical area in rat kidney with chronic pyelonephritis 30 days after IB inoculation with E. coli. Photomicrograph: H&E × 185. Fibrosis of Bowman's capsule is present (arrows), as well as interstitial leukocytic infiltration and dilated tubules. This kidney was bacteriologically sterile and from a nonmedicated animal.*

of positive bacterial cultures was usually less than that of pyelitis lesions, except in 2-day IC kidneys.

*Effect of chemotherapy on kidney lesions in rats.* The data in Tables 2 and 3 were analyzed with the Rank-sum Test (Dixon and

Massey, 1957), by comparing the group given nitrofurantoin with each of the other two groups (nonmedicated control and chloramphenicol), in each of three experiments. Only in the first experiment (Table 2) were significant differences found; nitrofurantoin group pyelitis grades were significantly less severe, at the 95% level, from both nonmedicated controls and chloramphenicol.

TABLE 2. *Histological evaluation of Escherichia coli pyelitis in rats following 2-day chemotherapy trials*

| Drug | Total kidneys | Severity of pyelitis† | | | | | |
|---|---|---|---|---|---|---|---|
| | | 0 | 1 | 2 | 3 | 4 | 5 |
| Nitrofurantoin* | 11 | 2 | 6 | 3 | - | - | - |
| Chloramphenicol* | 12 | 2 | 2 | 2 | 6 | - | - |
| Control | 10 | - | 1 | 2 | 5 | - | 2 |

* Four doses at 10 mg/kg; first dose 6 hr after IB inoculation.
† Symbols: 0 =no pyelitis; 1 to 5 = increasing severity of pyelitis.

TABLE 3. *Histological evaluation of Escherichia coli pyelonephritis in rats following chemotherapy trials*

| Severity of lesions* | | IB Inoculation day 0 10 mg/kg/day, day 2 to 10 | | | IC Inoculation day 0 10 mg/kg/day, day 2 to 10† | | |
|---|---|---|---|---|---|---|---|
| | | Control | Nitro-furantoin | Chloram-phenicol | Control | Nitro-furantoin | Chloram-phenicol |
| Pyelitis | 0 | - | - | 2 | 5 | 6 | 8 |
| | 1 | 2 | 1 | 7 | 1 | 3 | 2 |
| | 2 | 2 | 2 | 2 | 3 | - | - |
| | 3 | 5 | 3 | 1 | 1 | 3 | 3 |
| | 4 | 2 | 1 | 2 | 1 | 1 | 4 |
| | 5 | 1 | 1 | 2 | - | - | 1 |
| Total kidneys | | 12 | 8 | 16 | 11 | 13 | 18 |
| Pyelonephritis | 0 | 10 | 7 | 13 | 5 | 7 | 16 |
| | 1 | - | - | - | 2 | 3 | - |
| | 2 | 1 | 1 | - | 1 | - | 2 |
| | 3 | - | - | - | 1 | 2 | - |
| | 4 | - | - | 2 | - | - | - |
| | 5 | 1 | - | 1 | 2 | 1 | - |
| Total kidneys | | 12 | 8 | 16 | 11 | 13 | 18 |

* Symbols: 0 = no lesion; 1 to 5 = increasing severity of lesions.
† This group was held for 10 days after last dose before autopsy.

## Discussion

The significance of pyelitis as an important factor in human pyelonephritis and experimental retrograde pyelonephritis has been stressed by previous observers (Andersen and Jackson, 1961). Our

findings confirm their concept of experimental retrograde infections, but in addition show that a significant, though lesser, number of hematogenous *E. coli* infections also result in pyelitis alone, as well as pyelonephritis.

The therapeutic effect of drugs in experimental pyelonephritis is more evident from bacteriological data showing eradication of bacteria from the kidney (Pitsch, Hebert, and Carey, 1962) than from histological data on the incidence and severity of lesions. These lesions persist in kidneys from which bacteria have been eradicated; how long they persist is not yet known.

Medication with nitrofurantoin reduces severity of experimental renal lesions if treatment is begun 6 hr after IB inoculation (Table 2). When nitrofurantoin treatment is begun 2 days after inoculation, severity of renal lesions is unaffected (Table 3). Therefore, histological observations support the finding (Pitsch, Hebert, and Carey, 1962) that early treatment of experimental kidney infection is more effective than delayed treatment.

## Acknowledgments

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## Literature Cited

Armed Forces Institute of Pathology. 1957. Manual of histologic and special staining techniques. Armed Forces Institute of Pathology, Washington, D. C.

Andersen, B. R., and G. G. Jackson. 1961. Pyelitis, an important factor in the pathogenesis of retrograde pyelonephritis. J. Exptl. Med. 114:375-383.

Dixon, W. J., and F. J. Massey. 1957. Introduction to statistical analysis, p. 289, 2nd ed. McGraw-Hill, New York.

Pitsch, B., T. Hebert, and W. F. Carey. 1962. Establishment and treatment of experimental *Escherichia coli* pyelonephritis. Antimicrobial Agents and Chemotherapy - 1961, p. 54-60.

# RELATION OF CLUMPING FACTOR PRODUCED BY STAPHYLOCOCCI TO THEIR PHAGOCYTOSIS AND INTRACELLULAR SURVIVAL

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The uniformity with which pathogenic staphylococci produce coagulase has made it seem likely that, in some way, the action of this enzyme must be related to pathogenicity. In this regard, coagulase-positive staphylococci have been found to be ingested more slowly by phagocytic cells in the presence of fibrinogen than are coagulase-negative strains. Moreover, once ingested, they are better able to survive within human polymorphonuclear cells than are coagulase-negative strains or than many other common bacterial pathogens. Thus, it is suggested that the efficiency of the phagocytic system may be poor with respect to coagulase-positive staphylococci, and that this in turn is a factor determining pathogenicity.

## Materials and Methods

In testing for coagulase production the simple slide test, which depends on clumping of staphylococci in plasma, has been widely adopted. It has been recognized for many years, however, that this does not specifically test for coagulase, or at least that discrepancies exist between this and the test tube method. In 1954 Duthie reported a detailed study of what he called "clumping factor" (or bound coagulase) and clearly identified this as a factor separate from coagulase itself. Since then, several studies of interest concerning the "clumping factor" have appeared. Most significant of these are the observations of Alami and Kelly (1959) that staphylococci, which produce either clumping factor or coagulase, but not both, can be isolated and are readily identified on semisolid medium containing plasma. They have also reported (Alami and Kelly, 1960) that strains which produce coagulase but not clumping factor are more pathogenic for mice than are other staphylococci. Moses and Hunt (1958) have reported studies on one such strain, the Smith strain. They noted an enhanced capacity of the virulent or "diffuse" colony type to multiply

within the mouse peritoneal cavity. Rogers and Melly (1960) have reported one study with the Smith strain and noted that it was more slowly phagocytized by human polymorphonuclear cells than the more common strains, but thought it otherwise similar with respect to total phagocytosis and intracellular survival.

The striking pathogenicity for mice of the clumping factor-negative, coagulase-positive strains made it seem of interest to study the interrelation of these strains and the phagocytic cells, especially since previous studies of this type with staphylococci had not taken into account the fact that clumping factor and coagulase are separate products of these microorganisms.

The present study reports observations on the phagocytosis of coagulase-positive, clumping factor-negative strains of staphylococci and their intracellular survival in polymorphonuclear leukocytes of humans and of mice. A comparison has been made of the following groups of staphylococci: (i) the common strains which produce both coagulase and clumping factor; (ii) four strains which produce clumping factor but not coagulase (two of these were obtained from Kelly at the University of Oklahoma Medical School and two were isolated in our laboratory); and (iii) five strains which produce coagulase but not clumping factor (one of these is the Smith strain, two were obtained from Kelly, and two were isolated in our laboratory). Since individual strains within these groups gave similar results, the results will be reported together.

## Results

Figure 1 shows the different colony types in soft agar medium containing plasma. "A" is a strain which produces only coagulase. The halo of coagulated plasma may be seen about the colonies. The absence of clumping results in an elongated colony. "B" is a strain which produces only clumping factor. The colony is compact but has no halo. "C" is a strain which produces both substances. Thus, it is compact and also has a halo of coagulated plasma. "D" is a strain which produces neither coagulase nor clumping factor and is included for comparison.

The studies of Alami and Kelly (1960) on pathogenicity for mice of the clumping factor-negative, coagulase-positive strains have been confirmed and will not be detailed here.

Figure 2 shows the activity of clumping factor and coagulase in the plasma of four animal species. The test strains are divided into three groups, the first being the common strains of *Staphylococcus aureus* which clump in human plasma but do not clot this plasma. Group 2 are unusual strains which clump in human plasma, but do not clot the plasma. These are of the same virulence for mice as Group 1. Group 3 coagulate human plasma, but do not clump, and

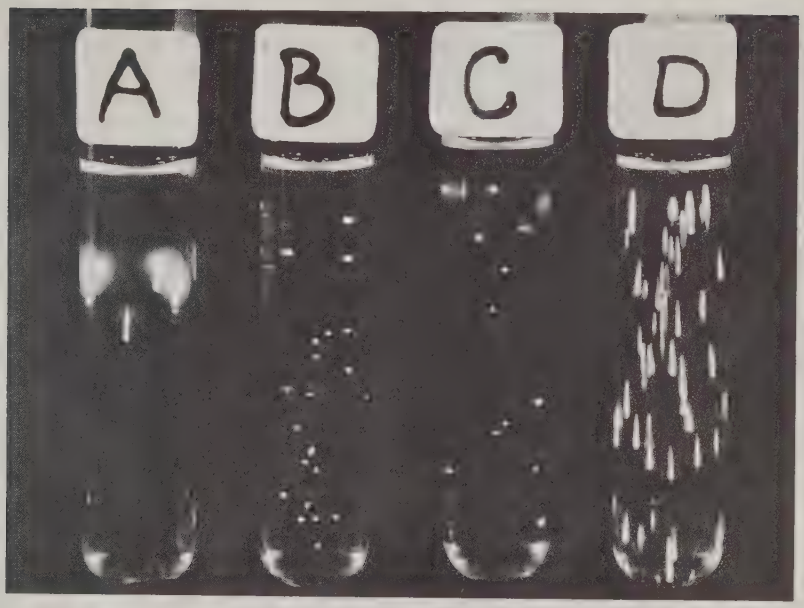


FIG. 1. *Colony types of staphylococci in soft agar medium containing plasma.* (A) *Produces coagulase.* (B) *Produces clumping factor.* (C) *Produces both coagulase and clumping factor.* (D) *Produces neither coagulase nor clumping factor.*

| | TEST STRAINS OF STAPHYLOCOCCUS | PLASMA | | | |
|---|---|---|---|---|---|
| | | HUMAN | MOUSE | RABBIT | GUINEA PIG |
| COAGULASE | GROUP 1 | + | O | + | + |
| | GROUP 2 | O | O | + | O |
| | GROUP 3 | + | O | + | + |
| CLUMPING FACTOR | GROUP 1 | + | + | + | O |
| | GROUP 2 | + | + | + | O |
| | GROUP 3 | O | O | O | O |

FIG. 2. *The activity of Staphylococcus clumping factor and coagulase in plasma of various animal species.*

are more highly virulent for mice. It is important to note that the classification used in this report is based on the behavior in human plasma for these reasons: none will coagulate plasma of the mice used in these studies; all coagulate rabbit plasma; none clump in guinea pig plasma.

It must also be added that the coagulase tests in guinea pig plasma as reported here are done at 25 C. Results at 37 C are erratic and often do not correspond with those noted here. In the following studies, therefore, the cultures will be designated with respect to the activity of coagulase and clumping factor in human plasma.

The details of the techniques employed in this study have been reported elsewhere (Rogers and Tompsett, 1952). Phagocytosis was studied by a simple roller-tube technique, utilizing cells obtained from human blood by sedimentation or from mouse peritoneal exudates. Intracellular survival was estimated by a slide culture technique.

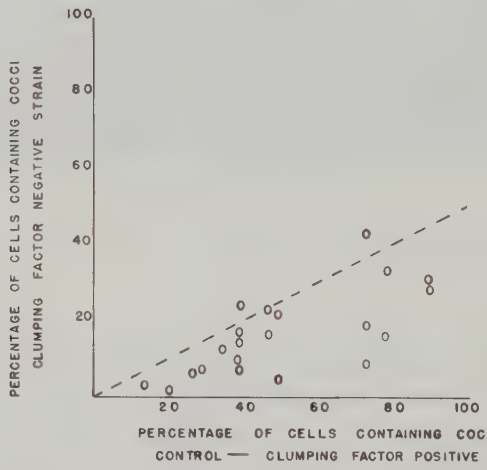


FIG. 3. *Comparative phagocytosis of clumping factor-negative and clumping factor-positive staphylococci by human polymorphonuclear cells.*

In Fig. 3 is shown the comparative phagocytosis of clumping factor-negative and clumping factor-positive strains. Since the absolute values can be altered significantly by variations in numbers of cells and bacteria, each result is plotted against its respective control. On the ordinate is the percentage of leukocytes containing clumping factor-negative staphylococci plotted in each case against the corresponding control on the abscissa, a clumping factor-positive strain. Values that fall below the broken line represent instances in

which phagocytosis of the clumping factor-negative strain was less than half of that of the clumping factor-positive control. As may be seen, phagocytosis of the clumping factor-negative strains was distinctly less than that of the control strain. These experiments represent only phagocytosis after 15 min of exposure. Increase in the contact time increases the degree of phagocytosis to some extent, but this same relationship holds up to 2 hr.

The ability of these strains to survive within leukocytes after intracellular residence of 1 to 4 hr was also tested (Fig. 4). After 1 hr of intracellular residence, survival of all types of staphylococci was relatively good, but the survival of clumping factor-negative strains (indicated by the open circles) was at least as good and frequently better than that of other strains. Similar results are plotted for intracellular residence of 4 hr. As may be seen, the comparatively better survival of the clumping factor-negative strains is more evident at this time.

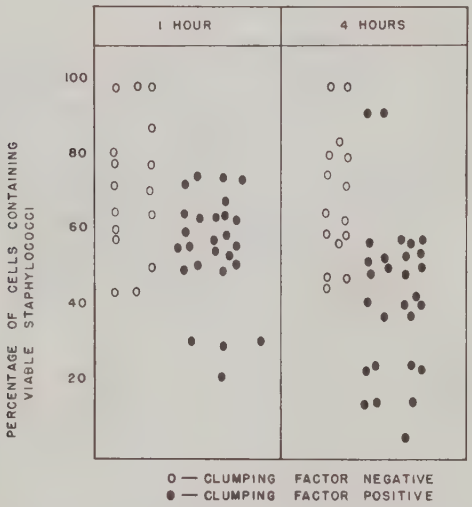


FIG. 4. *Comparative survival of clumping factor-negative and clumping factor-positive staphylococci in human polymorphonuclear cells.*

The same parameters were then tested in mouse leukocytes obtained from peritoneal exudates. Figure 5 depicts the comparative phagocytosis of the two types of staphylococci. Again, as in the human cells, phagocytosis of clumping factor-negative strains was generally less than 50% of that of clumping factor-positive strains during this 15-min test period. In the case of mouse cells, as opposed to human cells, more prolonged exposure resulted in an increase

in the relative percentage of cells which were phagocytic, so that after 1 to 3 hr the percentage approximated that of the clumping factor-positive strains.

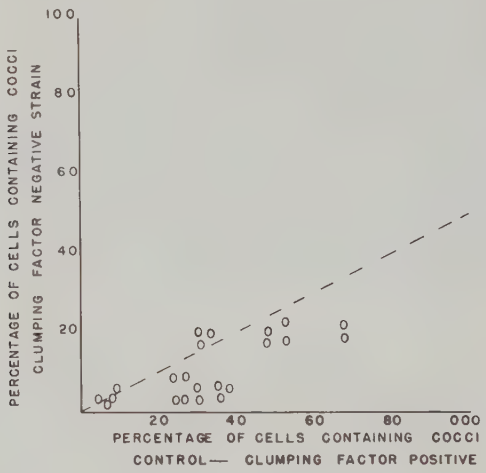


FIG. 5. *Comparative phagocytosis of clumping factor-negative and clumping-factor positive staphylococci by mouse polymorphonuclear cells.*

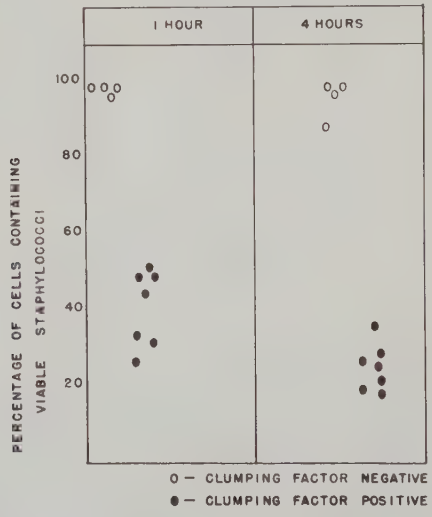


FIG. 6. *Comparative survival of clumping factor-negative and clumping factor-positive staphylococci in mouse polymorphonuclear cells.*

In Fig. 6 are shown results of experiments testing the ability of staphylococci to survive within mouse polymorphonuclear cells. It may be readily seen that coagulase-positive, clumping factor-negative strains survive distinctly better after both 1 and 4 hr intracellular residence than do strains which produce clumping factor.

Studies with cells of the rabbit and the guinea pig are as yet incomplete and will be reported at a later date.

## Acknowledgments

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## Literature Cited

Alami, S. Y., and F. C. Kelly. 1959. Demonstration of staphylococcal clumping factor and free coagulase in soft agar media. J. Bacteriol. **78**:539-544.

Alami, S. Y., and F. C. Kelly. 1960. Influence of coagulase and route of injection on staphylococcal virulence in mice. Proc. Soc. Exptl. Biol. Med. **105**:589-591.

Duthie, E. S. 1954. Evidence for two forms of staphylococcal coagulase. J. Gen. Microbiol. **10**:427-436.

Hunt, G. A., and A. J. Moses. 1958. Acute infection of mice with Smith strain of *Staphylococcus aureus*. Science **128**:1574-1575.

Rogers, D. E., and M. A. Melly. 1960. Further observations on the behavior of staphylococci within human leukocytes. J. Exptl. Med. **111**:533-558.

Rogers, D. E., and R. Tompsett. 1952. The survival of staphylococci within human leukocytes. J. Exptl. Med. **95**:209-230.

# COMPARATIVE EFFICACY OF VARIOUS ANTIBACTERIALS IN NORMAL AND IN IMMUNOLOGICALLY DEFICIENT MICE

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Although there is some general acceptance that host defenses have a complementary function in the chemotherapy of bacterial infections, this kind of interrelationship is suspected more than it is understood. In his critique, "The Forgotten Host," Jawetz (1955) properly emphasized immunologic intervention, and he logically suggested that a bactericidal agent would be less dependent upon host factors than a bacteriostatic one. Indeed, a cursory examination of some reported experiments in this area would appear to support this supposition on bactericidal vis-a-vis bacteriostatic agents (Mogabgab and Thomas, 1952; Miller et al., 1952; Kaplan, Speck, and Jawetz, 1952; Ilavsky and Foley, 1953; Gowans, 1953; and Jawetz, 1954).

The experiments to be described in this communication are, in part, a more critical inquiry on the comparative behavior of presumed bactericidal and bacteriostatic substances. Also, this study is a preliminary venture into the broader question of relationships among the microbe, host, and drug, wherein variation is applied to each of the three entities.

## Materials and Methods

*Bacteria: Streptococcus pyogenes*, C203 strain, *Diplococcus pneumoniae*, SVI strain, and *Klebsiella pneumoniae*, AD strain, were selected primarily because they have a high degree of intrinsic infectivity for mice. Mouse virulence was maintained either by weekly passage through mice or by the use of lyophilized cultures prepared from isolates recently passed through mice. Suspensions for challenge were derived from 6-hr growth at 37 C in Trypticase soy broth (Baltimore Biological Laboratories) and diluted in this broth in appropriate tenfold decrements. In all treatment experiments, mice were challenged with an estimated 100 $LD_{50}$ of each organism, injected intraperitoneally in 0.5 ml of diluent. This dose in normal mice effected a group mortality of at least 90% within 48 hr.

*Mice.* Carworth Farms "CF-W" mice, 18 to 22 g, both male and female, were used throughout. They were housed in groups of 15 to 20 in rectangular glass jars, and, unless otherwise specified, received a pellet ration and water.

*Resistance depression.* Two procedures were applied to increase the susceptibility of mice to infection by the previously named bacteria: The challenge organism was injected in a 5% hog gastric mucin vehicle (Wilson Laboratories, Type 1701-W), or the mice were starved for 40 hr prior to challenge. Starvation involved denial of food, but not water, up through the time of challenge; food pellets were made available within an hour after challenge and thereafter.

*Treatment.* Because the course of disease was of relatively short duration, especially in depressed mice, treatment was acute and decisive, as a rule, within 24 hr. Therefore, all antibacterials were given as a single dose at the time of challenge. We also believed that distinctions between bacteriostatic and bactericidal effects would be more clearly demonstrable from a single dose regimen.

Chloramphenicol and sulfadiazine were given in varying dosage levels as 0.5 ml per mouse in 5% aqueous gum acacia by oral intubation. Penicillin G, streptomycin, and neomycin were dissolved in saline at appropriate concentrations, and were injected subcutaneously as 0.5 ml per mouse. These antibacterials were freshly prepared prior to use. All these agents were made up in twofold decrements, and groups of 20 mice were used for each dose level.

To provide uniformity for comparison, a given experiment involving one drug and one bacterial species was performed simultaneously. For example, groups of normal, starved, and mucinized mice were challenged with *S. pyogenes* and treated with penicillin G, at varying dose levels within each group, on the same day. All experiments were run at least twice, and survival data taken 14 to 21 days after challenge were recorded. The effectiveness of a given antibacterial against a specific organism for normal and depressed mice was expressed in terms of the 50% effective dose ($ED_{50}$) estimated by the method described by Miller and Tainter (1944).

## Results

*Challenge resistance.* The comparative susceptibility of normal and depressed mice to each challenge organism was examined preliminary to treatment experiments. The $LD_{50}$ data in Table 1, although they are approximations, indicate that starved and mucinized mice were of increased susceptibility to the *Streptococcus* and *Klebsiella* challenges, but not so to the pneumococcus. In all three infections, however, we noted that the time to death was considerably decreased in the starved and mucinized animals. It is also noteworthy that although 40 hr of starvation caused about a 30% weight

loss, paper strip electrophoretic analyses of serum from starved mice did not reveal a state of general hypoproteinemia or of specific alterations in serum protein fractions.

TABLE 1. *Challenge susceptibility of normal and depressed mice*

| Challenge organism | Approx. number of cells $LD_{50}$ | | |
|---|---|---|---|
| | *Normal** | *Starved†* | *Mucinized** |
| *D. pneumoniae* (SVI) | 20 | 10 | 30 |
| *S. pyogenes* (C203) | 150 | 70 | 50 |
| *K. pneumoniae* (AD) | 400 | 90 | 70 |

*Mice in this group weighed 18 to 22 g at challenge.
†Mice in this group weighed 12 to 14 g at challenge.

*Streptococcus experiments.* Penicillin G proved to be about as effective in starved mice as it was in normal mice (Table 2). In contrast, however, nearly 20 times as much penicillin G was required to control this streptococcus infection in mucinized mice as compared to normal controls.

TABLE 2. *Comparative effectiveness of various antimicrobials against Streptococcus pyogenes in normal and depressed mice*

| Agent | Route | $ED_{50}$ | | |
|---|---|---|---|---|
| | | Normal | Starved | Mucinized |
| | | *mg/kg* | *mg/kg* | *mg/kg* |
| Penicillin G | Subcut. | 0.10 ± 0.06 | 0.15 ± 0.05 | 1.9 ± 0.7 |
| Sulfadiazine | Oral | 30 ± 3 | 280 ± 70 | 500 ± 140 |
| Chloramphenicol | Oral | 57 ± 4 | 72 ± 7 | 125 ± 18 |

That sulfadiazine fared poorly in depressed mice was shown by the fact that a nearly tenfold higher dose level than normal was needed for treating starved mice and an even greater amount for the mucinized mice.

Chloramphenicol activity was not markedly different between the three groups of mice. About twice as much of this antibiotic was required for effectiveness in mucinized mice, as compared to normal mice, and, as previously noted for penicillin G, the activity of chloramphenicol was roughly the same in starved and in normal animals.

*Pneumococcus experiments.* The most provocative observation made in these tests was that sulfadiazine showed distinct activity in mucinized mice, whereas it was essentially ineffective in normal and in starved mice (Table 3). Penicillin G proved about one-fourth as

active in depressed mice as compared to normal ones. Chloramphenicol activity, relative to normal mice, was somewhat lessened in starved animals and slightly greater in the mucinized group.

TABLE 3. *Comparative effectiveness of various antimicrobials against Diplococcus pneumoniae in normal and depressed mice*

| Agent | Route | $ED_{50}$ | | |
|---|---|---|---|---|
| | | Normal | Starved | Mucinized |
| | | *mg/kg* | *mg/kg* | *mg/kg* |
| Penicillin G | Subcut. | 4 ± 0.5 | 17 ± 4 | 18 ± 3 |
| Sulfadiazine | Oral | >1000 | >1000 | 410 ± 18 |
| Chloramphenicol | Oral | 410 ± 38 | 570 ± 64 | 320 ± 50 |

*Klebsiella experiments.* Again, as noted in the antipneumococcal tests, sulfadiazine was considerably more effective against *Klebsiella* in mucinized mice than it was in normal and in starved mice (Table 4). Streptomycin and neomycin were nearly equal, and the requirement for both was substantially increased in the mucin-treated mice. Slightly more chloramphenicol was required in starved mice and three times more than normal was needed in the mucinized mice.

TABLE 4. *Comparative effectiveness of various antimicrobials against Klebsiella pneumoniae in normal and depressed mice*

| Agent | Route | $ED_{50}$ | | |
|---|---|---|---|---|
| | | Normal | Starved | Mucinized |
| | | *mg/kg* | *mg/kg* | *mg/kg* |
| Streptomycin | Subcut. | 1.6 ± 0.2 | 3.1 ± 0.6 | 7.5 ± 1.5 |
| Neomycin | Subcut. | 1.5 ± 0.2 | 1.2 ± 0.4 | 6.5 ± 0.1 |
| Sulfadiazine | Oral | 460 ± 190 | 430 ± 170 | 75 ± 12 |
| Chloramphenicol | Oral | 26 ± 4 | 41 ± 5 | 77 ± 8 |

## Discussion

These experiments are only a small entry into a large area and are limited by our lack of knowledge of underlying mechanisms. Although we may infer from our test results, as well as from the reports of others (Olitski, 1948; Smith and Dubos, 1956; McGuire and Floyd, 1958), that mucin or starvation depresses the infection resistance of mice, we do not know how this depression is accomplished. Despite this limitation, we may proceed on the premise that our experiments involved two different populations of mice: one normal

and the other deficient in resistance. We may also conclude that the effectiveness of a given drug against a given infection was usually different in the two types of mice. The degree of this difference, however, was not predictably related to the "bactericidalness" or "bacteriostaticness" of the antibacterial agent.

Penicillin G, for example, is widely regarded as a highly bactericidal agent for actively multiplying group A streptococci. This quality suggests that penicillin G would be largely independent of host factors in eradicating such streptococci. That this premise may be questionable can be adduced by our observation that this antibiotic was substantially less effective against coccal challenges in depressed mice. Similar observations on penicillin have been made in experiments with leukopenic and cortisone-treated mice (Gowans, 1953; Jawetz, 1954).

Conversely, and notwithstanding their classification as bacteriostatic agents, both chloramphenicol and sulfadiazine did not appreciably lose effectiveness when given to infected depressed mice. Indeed, chloramphenicol was in several instances about as active in deficient mice as it was in normal ones. The surprising observation that sulfadiazine showed improved activity in mucinized mice is not readily explainable. We suspect that mucin interference of phagocytosis and resultant exposure of the bacteria to drug action may be a basis for the sulfadiazine anomaly.

On the basis of these experiments alone, we cannot propose a more rational basis for chemotherapy than the existing procedures which have been derived empirically. The extension of this kind of research may ultimately designate which agent is most likely to be successful against a given microbe in a particular state of immune deficiency. For the present we would submit, in agreement with MacLeod's postulate (1955), that perfect or absolute bactericidal action in vivo is not attainable under the usual regimens of systemic chemotherapy.

## Literature Cited

Gowans, J. L. 1953. The effect of some antibiotics on coccal infection in mice. Brit. J. Exptl. Pathol. **34**:195-201.

Ilavsky, J., and E. J. Foley. 1953. Observations on antibiotic treatment of bacterial infections of cortisone-treated mice. Proc. Soc. Exptl. Biol. Med. **84**:211-214.

Jawetz, E. 1954. Effect of cortisone on therapeutic efficacy of antibiotics in experimental infections. A. M. A. Arch. Internal Med. **93**:850-862.

Jawetz, E. 1955. The forgotten host. Stanford Med. Bull. **13**:84-90.

Kaplan, H. S., R. S. Speck, and E. Jawetz. 1952. Impairment of antimicrobial defenses following total body irradiation of mice. J. Lab. Clin. Med. **40**:682-691.

MacLeod, C. M. 1955. Interactions of host, microbe, and chemotherapeutic agent. Bull. N. Y. Acad. Med. **31**:427-437.

McGuire, C. D., and T. M. Floyd. 1958. Studies on experimental shigellosis. II. The effect of fasting and fatigue on *Shigella flexneri* 3 infections in mice. J. Exptl. Med. **108**:277-282.

Miller, C. P., C. W. Hammond, M. Tompkins, and G. Shorter. 1952. The treatment of postirradiation infection with antibiotics; an experimental study on mice. J. Lab. Clin. Med. **39**:462-479.

Miller, L. C., and I. M. Tainter. 1944. Estimation of the $ED_{50}$ and its error by means of logarithmic-probit graph paper. Proc. Soc. Exptl. Biol. Med. **57**:261-264.

Mogabgab, W. J., and L. Thomas. 1952. The effects of cortisone on bacterial infection. J. Lab. Clin. Med. **39**:271-289.

Olitski, L. 1948. Mucin as a resistance-lowering substance. Bacteriol. Rev. **12**:149-172.

Smith, J. M., and R. J. Dubos. 1956. The effect of nutritional disturbances on the susceptibility of mice to staphylococcal infections. J. Exptl. Med. **103**:109-118.

# LONG-TERM, LOW-LEVEL ANTIBIOTICS AND THE EMERGENCE OF ANTIBIOTIC-RESISTANT BACTERIA IN HUMAN VOLUNTEERS

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The effectiveness of nonmedical uses of antibiotics in plant disease control and food preservation has been recently reviewed in detail (Goodman, 1959; Wrenshall, 1959). The delay in widespread adoption of antibiotics for these uses, particularly in food preservation, has been caused by intelligent concern about potential hazards to the public health, primarily with regard to antibiotic-resistant bacteria. However, since resultant antibiotic residues are usually about 100- to 1,000-fold less than a therapeutic dose, it has been suggested that emergence of antibiotic-resistant bacteria may not be significant at this level. Studies with experimental animals were carried out in attempts to examine this problem. In 1958 it was reported (Goldberg, Read, and Goodman, 1958) that feeding of streptomycin to experimental mice at low levels (20 to 40 μg/animal/day) for several months induced the emergence of streptomycin-resistant coliform bacteria. Since the percentage of resistant bacteria occurred at a significant rate and the level of resistance was as high as 10,000 μg/ml, it was concluded that a certain amount of public health hazard was potentiated by low-level chronic intake of streptomycin.

Some time later similar studies were carried out with chlortetracycline (CTC) (Goldberg, Goodman, and Lanning, 1959), since the tetracyclines appear to be the antibiotics of choice for food preservation, and clinical experience had indicated these antibiotics to be less of a problem than streptomycin when considering emergence of resistance. The data showed CTC did not cause detectable emergence of resistance by coliform bacteria. Some resistance did occur with lactose-negative enterics *(Proteus-Pseudomonas)*. However, these levels of resistance were minimal and the percentage of organisms exhibiting resistance was low, when compared to the streptomycin results.

In contrast to Goldberg et al. (1959), Smith and Crabb (1957) and others (Gordon, Garside, and Tucker, 1959), working in England, reported rapid development of coliform resistance in chickens and pigs fed CTC at low levels.

On the basis of these observations and the desire to learn more about the bacteriological effects of long-term, low-level tetracycline studies in humans, this investigation was undertaken.

## Materials and Methods

Test subjects were human, adult, male volunteers from the Missouri State Penitentiary. These subjects provided the advantage of similar diet, environment, age group, and ready access to health records. Originally, 50 men began the project and were divided into two groups of 25 each. However, at the final evaluation of the project only 46 individuals completed all phases; one group had 25 subjects, the other 21. One group was placed on a regimen of 10 mg oxytetracycline (OTC) daily for 6 months, the other on placebo (lactose), in capsule form, for a similar period. At the end of this period both groups were taken off further medication for a 4-month period. Subsequently, the groups were reversed with the former placebo group going on antibiotics for 6 months and the antibiotic group going on placebo. In this way each individual became his own control.

The concentration of OTC was determined by analysis of residues in foods preserved with OTC and CTC. Such foods as fish, fresh meats, and vegetables have anticipated residues of 1 to 10 ppm (Wrenshall, 1959). On this basis, the test subjects who usually consumed 1 to 2 kg of food daily were given antibiotics calculated to result in 5 to 10 ppm of their total diet.

*Sampling.* The following samples were taken from each subject, prior to any medication: fecal samples for coliform and yeast analysis and blood samples for OTC serum assay. In addition, each individual was skin tested with OTC for determination of hypersensitivity. After the initial establishment of base-line data, these tests were performed on a regular basis throughout the study. Bacteriological analysis was done monthly on samples from all subjects, as were blood-serum assays for OTC. On a bimonthly basis all individuals were skin tested for OTC hypersensitivity. The methods are described below.

*Bacteriology.* Each 30 days, fecal samples were collected from the test subjects. Samples (1 g) were homogenized in a Virtis homogenizing flask, diluted, and plated in triplicate on MacConkey agar (Difco) for coliform count and isolation. Dilutions were established to yield 50 to 200 colonies on MacConkey agar to replicate these colonies on subsequent MacConkey agar plates containing 10, 100, and 1,000 $\mu$g/ml OTC for resistance study. Incubation was at 24 to 48 hr at 37 C. The replication technique (Lederberg and Lederberg, 1952) obviated the necessity for multiple samples of fecal suspensions, and the resultant, inherent difficulties in obtaining uniformity. With replication it was possible to determine the percentage of coliforms,

from the original fecal samples, resistant to 10, 100, and 1,000 μg/ml OTC. Whenever the majority of colonies from the original non-antibiotic plates replicated to an OTC plate, it was assumed that the individual providing the original sample carried large numbers of coliforms resistant to that concentration of OTC.

Throughout the study single coliform colonies were picked and tested for OTC sensitivity by the gradient-plate technique (Braude, Banister, and Wright, 1955). In addition, representative isolated colonies were tested and identified as *Escherichia coli*, typed serologically by commercial typing sera, when possible. This was done to detect any serotype change accompanying emergence of resistance, as has been reported for enterococci following long-term, low-level CTC in chickens (Eliott and Barnes, 1959).

Quantitative yeast counts were made monthly on each individual by plating triplicate dilutions of homogenized fecal suspensions on Pagano's neomycin-TTC medium (Difco). Resultant yeast colonies were identified by varying intensity of red pigment and randomly checked microscopically to confirm yeast morphology. No attempt was made to establish the taxonomic status of the yeasts. These plates were incubated 48 to 72 hr at 30 C.

*OTC in serum.* Each individual provided a blood sample, for assay of OTC, once each month. The standard method described by Grove and Randall (1955) was followed. Using this technique, 0.025 μg/ml OTC can be detected.

*Skin test.* The development of hypersensitivity to OTC, induced by its long-term, low-level administration, was tested by an intradermal test using a 0.1 ml solution of OTC hydrochloride containing 1 mg/ml (Loughlin, Alcindor, and Joseph, 1958). This test was applied to each subject bimonthly.

## Results

Table 1 illustrates the effect of OTC on the total count of coliforms and yeasts, after 6 months of daily antibiotic intake. In nine of the 46 individuals, the antibiotic resulted in 10- to 100-fold increased

TABLE 1. *Effect of antibiotic treatment on median yeast and Escherichia coli counts*

| Organism | Maximum log increase in count | | | | Total |
|---|---|---|---|---|---|
| | <1* | 1 to 2 | 2 to 3 | >3 | |
| | *No. of persons* | | | | |
| *E. coli* | 37 | 9 | 0 | 0 | 46 |
| Yeast | 28 | 15 | 1 | 2 | 46 |

*Indicates variation in count (increase or decrease) was always less than 1 log.

coliform count. However, in the remaining 37 persons, the coliform count varied by less than 10-fold, and this variation was often a decrease rather than an increase.

The yeast count, however, shows a more consistent increase. Counts of two individuals were increased more than 1,000-fold and a total of 18 of the 46 persons showed increased yeast count after 6 months of OTC.

Serological typing of randomly selected *E. coli* colonies indicated the presence of types 0119 and 0125 in at least half of the test subjects at one sampling or another. However, these did not appear to be effected by the experiment in any way. They never were found in great numbers in any fecal samples, were always sensitive to OTC, and seemed to have no significance with regard to the study.

Preliminary OTC sensitivity testing of hundreds of coliform bacteria from the prisoners indicated that on the average they were sensitive to 3.0 μg/ml OTC by the gradient-plate technique using MacConkey agar. It was, therefore, arbitrarily established that the coliform organisms would be considered resistant if they could grow at 10 μg/ml OTC.

Appearance of OTC resistance became apparent by the end of the first month, although this by no means occurred in all the individuals on the antibiotic regimen. Table 2, for example, indicates that 10 persons in Group A and 11 in Group B showed OTC-induced resistance at the 10 μg-level. Table 3, at the 100-μg level, shows a correspondingly similar picture as does Table 4. In each instance, however, resistant coliforms were induced in less than half of the individuals participating.

TABLE 2. *Individuals with OTC-induced resistant (10μg/ml) coliforms at the end of the first month*

| | Group A | Group B | Total |
|---|---|---|---|
| Induced resistance | 10 | 11 | 21 |
| No induced resistance | 15 | 10 | 25 |
| Total | 25 | 21 | 46 |

TABLE 3. *Individuals with OTC-induced resistant (100μg/ml) coliforms at the end of the first month*

| | Group A | Group B | Total |
|---|---|---|---|
| Induced resistance | 10 | 8 | 18 |
| No induced resistance | 15 | 13 | 28 |
| Total | 25 | 21 | 46 |

If we look at the results for the six cumulative months on antibiotic, we observe a few more individuals who harbor resistant

coliforms (Tables 5 and 6). Most notable is the fact that resistance to 1,000 μg has not altered between the first and sixth months on antibiotic. Thus, Table 7 is the same as Table 4. Statistical analysis indicates the validity of these figures and is appended to Table 7. Once again, though, it is apparent that the highest level of OTC-induced resistance is limited to a minority of the participants.

TABLE 4. *Individuals with OTC-induced resistant (1,000 μg/ml) coliforms at the end of the first month*

| | Group A | Group B | Total |
|---|---|---|---|
| Induced resistance | 2 | 7 | 9 |
| No induced resistance | 23 | 14 | 37 |
| Total | 25 | 21 | 46 |

TABLE 5. *Individuals with antibiotic-induced resistance (10 μg/ml) following 6 months on OTC*

| | Group A | Group B | Total |
|---|---|---|---|
| Induced resistance | 15 | 10 | 25 |
| No induced resistance | 10 | 11 | 21 |
| Total | 25 | 21 | 46 |

TABLE 6. *Individuals with antibiotic-induced resistance (100 μg/ml) following 6 months on OTC*

| | Group A | Group B | Total |
|---|---|---|---|
| Induced resistance | 12 | 8 | 20 |
| No induced resistance | 13 | 13 | 26 |
| Total | 25 | 21 | 46 |

TABLE 7. *Individuals with antibiotic-induced resistance (1,000 μg/ml) following 6 months on OTC**

| | Group A | Group B | Total |
|---|---|---|---|
| Induced resistance | 2 | 7 | 9 |
| No induced resistance | 23 | 14 | 37 |
| Total | 25 | 21 | 46 |

*The following equations represent the statistical confirmation of the data obtained:

$$X^2 = \frac{[7 \times 12 - 2 \times 14]^2 (46)}{25 \times 21 \times 9 \times 37}$$

$$= \frac{[161 - 28]^2 (46)}{42,525} = \frac{813,694}{42,525} = 19.13$$ (Statistically significant at $P < .01$)

The final table on resistance (Table 8) compares the status of OTC-induced resistance with existing resistance uninfluenced by OTC. In addition, results are included which describe the OTC resistance in coliforms from volunteers 2 months after all medication and the cessation of the study. Even without antibiotic, while taking placebo, several individuals carried coliforms resistant to 10 and 100 μg, but never to 1,000 μg. Those individuals whose resistance was induced by OTC returned to normal within 2 months after antibiotic was stopped. The only individuals remaining with resistant coliforms were those who had this pattern irrespective of antibiotic.

TABLE 8. *Individuals with OTC-resistant coliforms at the close of the study*

| Resistant dose | Group A | | | Group B | | |
|---|---|---|---|---|---|---|
| | OTC induced | Placebo | 2 Months post medication | OTC induced | Placebo | 2 Months post medication |
| 10 μg/ml | 15 | 1 | 1 | 10 | 8 | 8 |
| 100 μg/ml | 12 | 1 | 1 | 8 | 5 | 5 |
| 1,000 μg/ml | 2 | 0 | 0 | 7 | 0 | 0 |

*Skin test.* While in the third month of OTC two individuals showed a positive skin test as manifested by a palpable wheal and erythema. No other effects were noted. These persons remained on OTC and subsequently showed negative reactions to further skin testing. Thus, of 400 skin tests done in this study, two were positive.

*OTC serum assay.* OTC level was tested by agar cylinder assay for a total of 800 assays. Antibiotic was not detected in any of the subjects' serum at any time. The 10 mg OTC/day routine was never enough to give detectable antibiotic in the blood serum.

*General health.* At no time during the 16 months of this study was there sign of other than general well-being in the volunteers. Yeast diseases such as thrush, glossitis, and pruritis failed to occur, as did any other overt manifestation of upset in the balance of microbial flora.

## Discussion

There are several instances in the literature of long-term, high-level uses of tetracycline antibiotics for therapeutic or prophylactic purposes. Shwachman and associates in several reports (1951, 1952, and 1958) have indicated use of chlortetracycline or oxytetracycline in treating chronic cystic fibrosis over a period of 8 to 9 years. These antibiotics were given in daily doses up to 25 mg/kg body weight. Although development of resistance by *Staphylococcus* and

*Proteus* species occurred and an increase in yeasts was found, the authors continued treatment and were satisfied with results, since only clinically beneficial responses were noted.

Sprunt and McVay (1953) used CTC at a daily level of 500 mg for 19 months in geriatric patients as a routine prophylactic. Toxic effects were minimal and did not require cessation of antibiotic. A reduction in respiratory and urinary infections was observed, although no studies on enteric microorganisms were carried out.

Other studies support the efficacy of therapeutic daily doses of tetracycline antibiotics as prophylactic agents over long periods. However, bacteriologic analyses were not reported (Stowens, 1951).

Studies of long-term, low-level tetracycline in humans are confined for the most part to attempts to correct growth failure in malnourished children. Joliffe et al. (1956) fed 20 mg CTC daily for 7 months to undernourished children. When compared with controls, these children showed greater weight gains. No untoward effects were reported, although no bacteriology was done. Similarly, Scrimshaw is reported by Carey (1958) to have fed 50 mg CTC daily to children for up to 30 months. No toxic effects were noted. Physical examination and hematological study supported this claim, although no bacteriology was done.

Bacteriologic analysis was carried out by Loughlin et al. (1958) in a study which evaluated OTC as a growth-promoting agent in 243 young children. Daily 50 and 10 mg doses were administered for 12 months to several groups of these children. Cultures of *S. aureus* and enterococci were isolated from rectal swabs and checked for OTC resistance prior to and periodically after the study began. A skin test was also employed to determine allergic sensitization to OTC. The results showed complete absence of toxic effects, gastrointestinal upsets, increased resistance, moniliasis, and positive skin tests.

The reports above on long-term tetracyclines at both high and low levels indicate the relative infrequency of undesirable effects from these antibiotics. Although instances of nausea, diarrhea, pruritis, and other minor side effects have been seen at long-term high dosage, these did not occur at low dosage.

Emergence of resistance has also been reported from high dosage, but, even here, antibiotic treatment problems failed to arise.

The low dosage studies, although lacking detailed bacteriological analysis, are noted for the absence of any side effects.

In this study, using 10 mg OTC/day in adults, it is clear that resistant coliforms and increased yeast count do occur in some individuals from the antibiotic. However, the facts that resistant coliforms exist prior to antibiotic administration, that OTC-induced resistance is transitory, and that, in the final analysis, general well-being was maintained by all volunteers indicate a doubtful public health hazard from long-term, low-level OTC. Absence of moniliasis,

lack of toxic effects, failure to induce permanent resistance or hypersensitivity, and the inability of this low dose of OTC to achieve a serum level seem to substantiate this statement.

In relating these findings to the problem at hand, the use of antibiotics in food preservation, there are two extremes of opinion to contend. It can be suggested that the question is entirely academic, since we may all be exposed to antibiotics in the consumption of foods like cheeses, sauerkraut, and fermented beverages. Indeed, it has been established that at least one antibiotic, nisin, is a natural component of many dairy foods (Mattick and Hirsch, 1944). On the other hand, there are those who feel that clinical experience has revealed serious untoward effects from antibiotics and that indiscriminate use in foods would extend the danger to the public. This group would prohibit the use of any and all antibiotics nonmedically.

Apparently, a middle course is the proper one, since it is well established that antibiotics behave differently from each other with regard to dosage, antimicrobial spectrum, and ability to induce resistance or allergic sensitization. It follows that each antibiotic or group of antibiotics should be evaluated separately for contemplated nonmedical uses. The well-documented ability of antibiotics to control plant disease, stimulate human and animal growth, and increase shelf-life of fresh and processed foods cannot be ignored.

## Acknowledgments

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The authors are indebted to Chas. Pfizer & Company for providing 10-mg capsules of oxytetracycline along with capsules containing placebo.

## Literature Cited

Braude, A. L., J. Banister, and N. Wright. 1955. Use of the gradient plate for routine clinical determinations of bacterial sensitivities to antibiotics. Antibiotics Ann. 1954-1955, p.1133.

Carey, B. W. 1958. Medical aspects of antibiotics for food preservation. Proc. Intern. Congr. Biochem. 5:208-218.

Elliott, S. D., and E. M. Barnes. 1959. Changes in serological type and antibiotic resistance of lancefield group D streptococci in chickens receiving dietary chlortetracycline. J. Gen. Microbiol. 20:426-433.

Goldberg, H. S., B. E. Read, and R. N. Goodman. 1958. Studies on the emergence of streptomycin-resistant bacteria as a result of low-level, long-term feeding of streptomycin. Antibiotics Ann. 1957-1958, p.144-148.

Goldberg, H. S., R. N. Goodman, and B. Lanning. 1959. Low-level, long-term feeding of chlortetracycline and the emergence of antibiotic-resistant enteric bacteria. Antibiotics Ann. 1958-1959, p.930-934.

Goodman, R. N. 1959. The influence of antibiotics on plants and plant disease control, p.322-448. *In* H. S. Goldberg, [ed.], Antibiotics their chemistry and non-medical uses. D. Van Nostrand Co., Inc., New York.

Gordon, R. F., J. S. Garside, and J. F. Tucker. 1959. Proc. XVIth Int. Vet. Cong., Madrid.

Grove, D. C., and W. A. Randall. 1955. Assay methods of antibiotics: a laboratory manual. Medical Encyclopedia, Inc., New York.

Jolliffe, N., G. Frontali, G. Maggioni, S. Carbo, and O. Lanciano. 1956. Effects of chlortetracycline on weight gain of Italian children ages 6 to 10 on diets relatively low in animal protein. Antibiotics Ann. 1955-1956, p. 19-26.

Lederberg, J., and E. M. Lederberg. 1952. Replica plating and indirect selection of bacterial mutants. J. Bacteriol. **63**:399-406.

Loughlin, Elmer H., L. Alcindor, and A. A. Joseph. 1958. Extended low-level dosage of oxytetracycline. Antibiotics Ann. 1957-1958, p.95-98.

Mattick, A. T. R., and A. Hirsch. 1944. A powerful inhibiting substance produced by group N streptococci. Nature **154**:551.

Shwachman, H., G. E. Foley, and C. D. Cook. 1951. Aureomycin in pediatrics. J. Pediat. **38**:91-127.

Shwachman, H., B. K. Silverman, P. R. Patterson, and L. J. Zheutlin. 1952. Antibiotics in treatment of pancreatic fibrosis, with emphasis on terramycin. J. Am. Med. Assoc. **149**:1101-1108.

Shwachman, H., and L. L. Kulczycki. 1958. Long-term study of one hundred five patients with cystic fibrosis. A. M. A. J. Diseases Children **96**:6-15.

Smith, H. W., and W. E. Crabb. 1957. The effect of the continuous administration of diets containing low-levels of tetracyclines on the incidence of drug-resistant *Bacterium coli* in the feces of pigs and chickens: the sensitivity of the *Bact. coli* to other chemotherapeutic agents. Vet. Record **69**:24.

Sprunt, D. H., and L. V. McVay, Jr. 1953. A long range study of the use of broad spectrum antibiotics in elderly people. Antibiotics Ann. 1953-54, p.273-278.

Stowens, Daniel. 1951. Aureomycin in the treatment of fibrocystic disease of the pancreas. Pediatrics **8**:60-67.

Wrenshall, C. L. 1959. Antibiotics in food preservation, p.449-527. *In* H. S. Goldberg, [ed.], Antibiotics their chemistry and non-medical uses. D. Van Nostrand Co., Inc., New York.

# NEWER ANTIBIOTICS AND MICROBIAL PERSISTENCE

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The persistence of antibiotic-sensitive microorganisms in infected tissues, despite therapy with antimicrobial agents active in vitro against the causative organisms, has been demonstrated in many disease states and represents a serious deterrent to effective treatment.

The phenomenon of microbial persistence was recognized early after the discovery of penicillin, although it undoubtedly existed prior to this time. Hobby, Meyer, and Chaffie (1942) reported that not all cells of a culture of hemolytic *Streptococcus* were killed in vitro by penicillin even though the progeny of the surviving cells were no more resistant than the parent strain. Bigger (1944) reported similar in vitro observations using staphylococci and first called these surviving bacteria "persisters." In 1945 the persistence of pneumococci was demonstrated in the nasopharynx of children during penicillin therapy (McDermott et al., 1958). Rammelkamp and Kirby (1945) made similar observations in the streptococcal carrier states. Two of the most striking clinical examples of the persistence of sensitive organisms were reported by Smadel et al. (1949). In these examples, chloramphenicol failed to eradicate the typhoid carrier state and did not prevent scrub typhus when given to exposed patients before the clinical symptoms appeared.

Much has been learned about the circumstances favoring the development of this "drug-indifferent" state. It has been clearly shown independently by McCune et al. (1957), Canetti (1955), Barkley et al. (1953), and Eagle (1952) that the failure of the antibiotic to come into contact with the infecting microorganisms is not the explanation for their survival. It has also been shown that dense bacterial populations were less susceptible to the action of penicillin than less dense concentrations (McDermott, 1958). Similarly, young populations were more susceptible than older populations. Eagle (1952) demonstrated this latter phenomenon in vivo by showing that streptococcal infections induced in mice were much more susceptible to treatment

with penicillin when treated within a few hours after inoculation than when treatment was withheld for 18 to 24 hr. Other observations, both experimental and clinical, indicate that the circumstances favoring the growth of bacteria and the development of a progressive infectious process also make the causative organism more susceptible to the effect of antibiotics. On the contrary, circumstances which are not favorable for the growth and spread of the infectious agent tend to produce the state of microbial persistence (McDermott et al., 1958).

In spite of the large number of observations made concerning the phenomenon of microbial persistence, little has been accomplished in arriving at a solution to the therapeutic problem created by the "drug-indifferent" state. It has been shown in vitro that certain factors, such as aeration, shaking, and adding nutrients, increase the susceptibility of persisters to antibiotics, but none of these offers a reasonable approach to the problem clinically.

## Clinical

Three years ago the role of microbial persisters as a cause of treatment failures became so impressive that an investigational program, aimed at a better understanding of this state, was inaugurated in this laboratory. Its importance was repeatedly demonstrated clinically by culturing antibiotic-sensitive strains of bacteria from patients during therapy with antibiotics active in vitro against the causative organisms of such diseases as acute staphylococcal endocarditis, chronic staphylococcal osteomyelitis, chronic staphylococcal nasal carrier states, chronic mastoiditis due to *Pseudomonas aeruginosa, Salmonella* septicemia, chronic prostatitis, infected vascular prostheses, and infected hematomas.

Persisters could be demonstrated most frequently from chronic localized infections, though, in some, the local infection served as a focus of a recurring bacteremia. A striking example of this is the case of a 45-year-old man who developed a staphylococcal endocarditis following surgery for the correction of stenosis of the aortic valve. He was treated postoperatively with chloramphenicol (Fig. 1). However, within a few days after the chloramphenicol was discontinued he became febrile and a coagulase-negative *Staphylococcus* was isolated from his blood stream (Fig. 2). The strain was found to be sensitive in vitro to most antibiotics including chloramphenicol. Results of the sensitivity tests using the bactericidal antibiotics are shown in Table 1.

Because of the seriousness of the disease and a recent history of a severe allergic reaction to penicillin, he was treated with both kanamycin and vancomycin. His blood cultures promptly became sterile, but within a few days after the antibiotics were discontinued

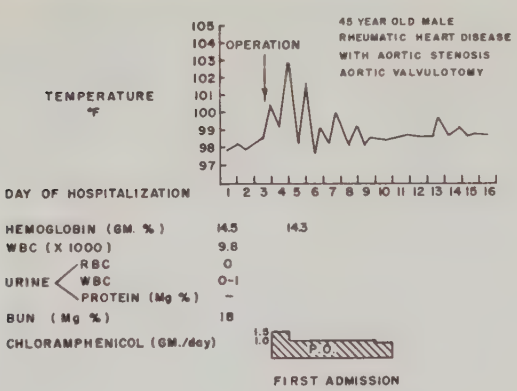


FIG. 1. *First hospital admission of case of staphylococcal endocarditis.*

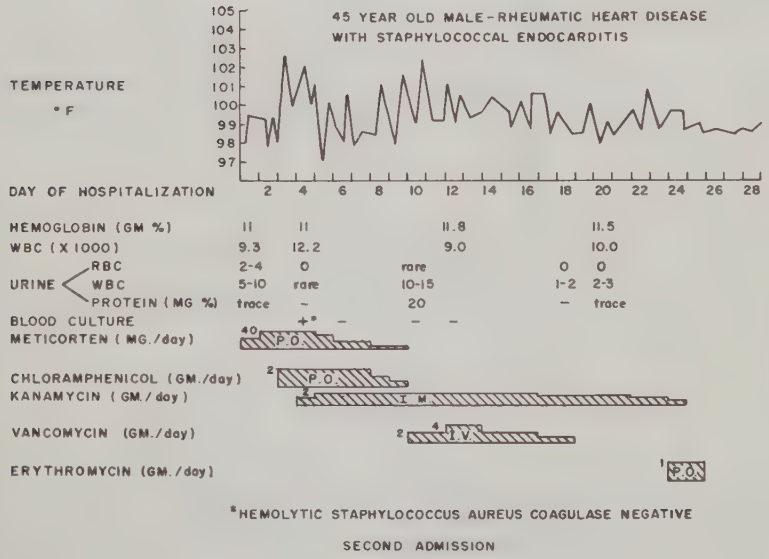


FIG. 2. *Second hospital admission of case of staphylococcal endocarditis.*

TABLE 1. *Sensitivity tests using bactericidal antibiotics*

| Antibiotic | Minimal inhibitory concentration | Minimal bactericidal concentration |
|---|---|---|
| | *μg/ml* | *μg/ml* |
| Penicillin G | 0.10 | 0.20 |
| Phenethicillin | 0.78 | 0.78 |
| Methicillin | 6.25 | 6.25 |
| Kanamycin | 0.20 | 0.78 |
| Vancomycin | 1.56 | 3.12 |
| Streptomycin | 20.0 | 50.0 |

and while he was still receiving erythromycin, his fever and bacteremia returned (Fig. 3). The strain of *Staphylococcus* isolated at this time was identical in all respects to the original strain. He was subsequently treated with methicillin and streptomycin. Cortisone was also given because of the evidence of history of allergy to penicillin. There was a prompt clinical and bacteriologic response, and after 30 days the treatment was discontinued.

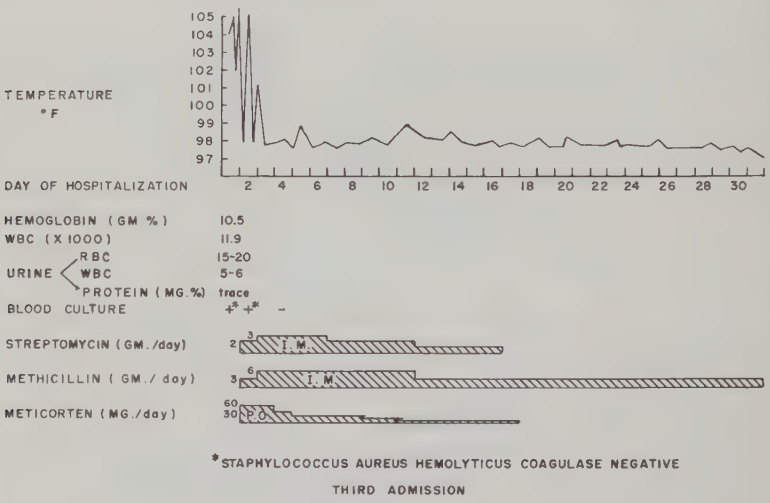


FIG. 3. *Third hospital admission of case of staphylococcal endocarditis.*

Three weeks later there was a relapse with a return of the fever, and a *Staphylococcus* with the same characteristics was isolated from the patient's blood (Fig. 4). The dose of methicillin was then increased to 22 g daily with 20 g administered by slow intravenous drip and 2 g rapidly given intravenously. The patient's serum showed a bactericidal effect against his organism in a dilution of 1:4.

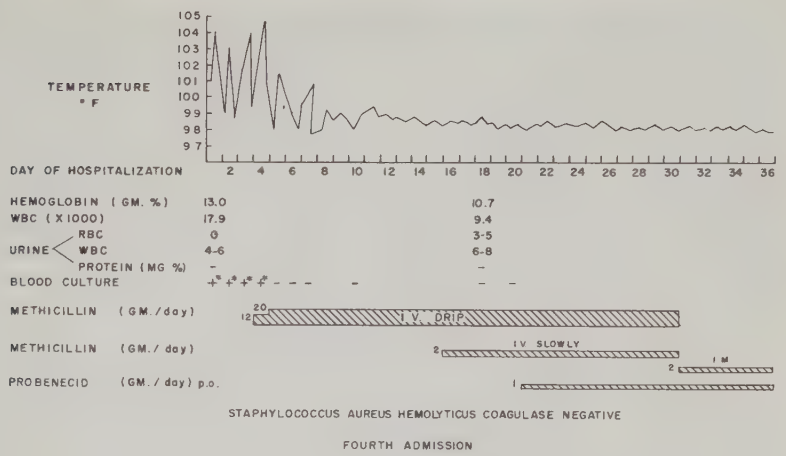


FIG. 4. *Fourth hospital admission of case of staphylococcal endocarditis.*

This regimen was continued for 4 weeks and followed by the intramuscular administration of 2.0 g daily for 4 weeks. Cessation of therapy was followed by another relapse, without evidence of increasing antibiotic resistance of the causative organism (Fig. 5). He was subsequently treated for 106 days with doses varying from 20 to 0.5 g daily. Although the smaller dose controlled the fever and bacteremia for 42 days, relapse ultimately recurred during therapy.

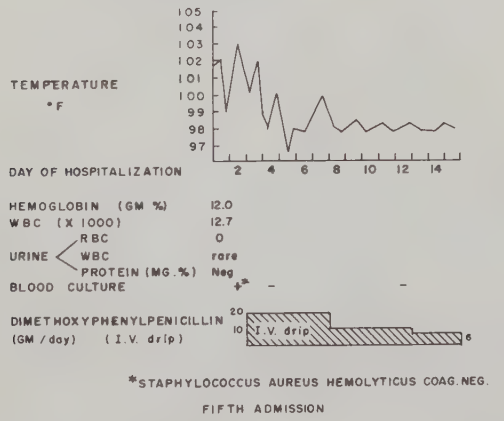


FIG. 5. *Fifth hospital admission of case of staphylococcal endocarditis.*

The patient was then placed on 45 g daily by intravenous drip (Fig. 6). There were no complications of therapy in this dosage range except for the development of a thrombophlebitis at the site of the infusions and excessive fluid retention as the result of the large amount of sodium administered in the methicillin salt. Each infusion was given over an 8-hr period to prevent the deterioration of the methicillin to the therapeutically inactive penicilloic acid. Measurement of the serum-killing power, during this course of methicillin, revealed that the serum killed the causative organism in dilutions of from 1:16 to 1:64. After 4 weeks of therapy, the drug was discontinued, and the patient has been observed for 1 year without evidence of recurrence of infection.

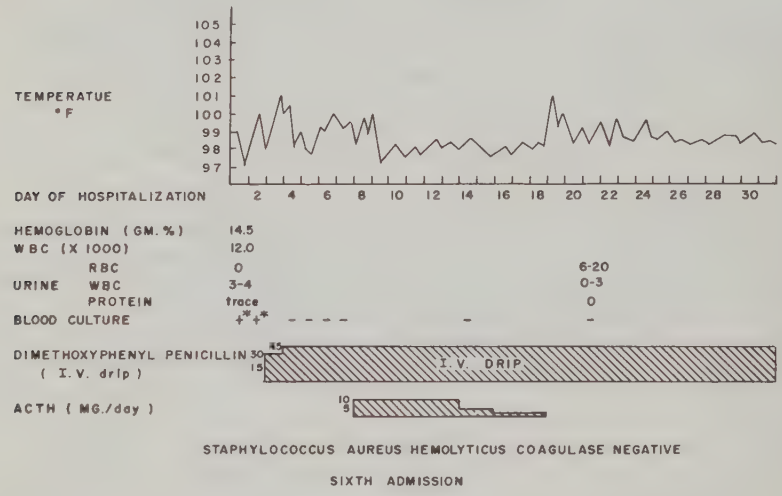


FIG. 6. *Sixth hospital admission of case of staphylococcal endocarditis.*

In some instances antibiotic-sensitive bacteria have been cultured from the blood stream during intensive therapy with one or more agents active in vitro against the organism producing the bacteremia. An example of this is that of a 34-year-old man admitted to the hospital for treatment of a compound fracture of the femur. Following surgery he was febrile and ultimately found to have a staphylococcal bacteremia (Fig. 7). Although he was treated with chloramphenicol, erythromycin, and bacitracin, antibiotics to which the staphylococcus was sensitive in vitro, his blood cultures remained positive and the patient expired.

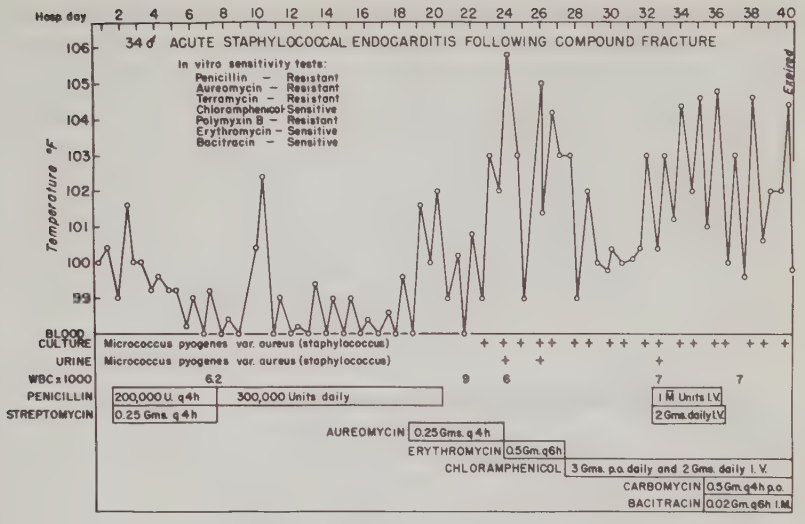


FIG. 7. *Summary of case of acute staphylococcal endocarditis following a compound fracture of femur.*

## Experimental

*Demonstrations of persisters in vitro.* Since most of the earlier studies dealing with microbial persistence used benzylpenicillin as the test antibiotic, experiments were designed to study this phenomenon with other more recently developed antibiotics.

A series of preliminary experiments were carried out demonstrating that, when a mature population of staphylococci ($10^8$ cells/ml) was exposed to kanamycin and methicillin, viable organisms could be recovered in concentrations of the antibiotics as great as 50 $\mu$g/ml. However, if the organisms isolated from the tubes containing high concentrations of the antibiotics were tested by conventional methods using $10^4$ cells/ml, they were found to be highly sensitive and as sensitive as the parent strain. The same culture was also found to be indifferent to the effect of kanamycin and methicillin when suspended in media (saline) not supporting growth.

A second group of experiments was designed to study, quantitatively, the survival of bacteria in the presence of varying concentrations of bacteria. These experiments were carried out by allowing a culture of *Staphylococcus* to mature by over-night incubation. Samples of the culture were diluted to allow final bacterial populations varying from $10^3$ to $10^8$ cells per ml. Each of these was then exposed to concentrations of antibiotics varying from 0.2 to 100 $\mu$g/ml.

After over-night incubation, the number of bacteria surviving each concentration of antibiotic was counted and tested for antibiotic sensitivity by the conventional tube-dilution method. These experiments were carried out using the following bactericidal antibiotics: penicillin, streptomycin, bacitracin, kanamycin, ristocetin, and vancomycin. The results indicated that antibiotic-sensitive bacterial persisters could be demonstrated with each antibiotic studied and that the number of persisters increased as the concentrations of the bacterial population increased, but decreased as the concentration of the antibiotic increased. Space does not permit a recording of the detailed results but these relationships are illustrated in the graph of the surviving bacteria exposed to varying concentrations of vancomycin (Fig. 8).

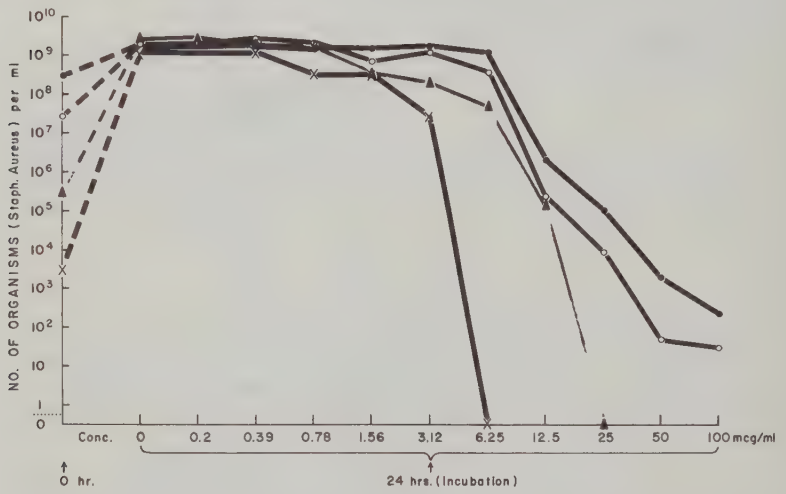


FIG. 8. *Effect of numbers of organisms on the antistaphylococcal activity of varying concentrations of vancomycin.*

*Demonstration of persisters in vivo.* A localized staphylococcal infection, with a predictable natural history, was produced in rabbits by the subcutaneous injection of 5 ml of human blood mixed with $10^5$ bacteria per ml. If a single injection of penicillin was given in appropriate amounts, immediately after the inoculation of the blood-bacteria mixture, and the animals sacrificed 5 days later, no abscesses could be found and negative cultures were obtained at the site of the inoculation. The results were the same if the injection was 1, 3, or 6 hr after infecting the animals, but at 12 and 24 hr and 5 days, doses as large as 500,000 units/kg failed to sterilize the abscesses, yet the bacteria cultured from the animals receiving these

TABLE 2. *Effect of single injection of penicillin G on cultures of abcesses taken 5 days later**

| Time of injection following inoculation | Sterilized | Number negative cultures | Number positive cultures |
|---|---|---|---|
| *hr* | % | | |
| Immediate | 92 | 11 | 1 |
| 1 | 92 | 11 | 1 |
| 3 | 84 | 10 | 2 |
| 6 | 84 | 10 | 2 |
| 12 | 0 | 0 | 12 |
| 24 | 21 | 4 | 17 |
| 5 days | 11 | 2 | 16 |
| Untreated controls | 27 | 3 | 9 |

*Penicillin G dosage: 5,000 to 500,000 units/kg.

large doses showed no change in sensitivity to penicillin in vitro (Table 2). Similar studies with erythromycin, tetracycline, novobiocin, chloramphenicol, kanamycin, and methicillin revealed persistence of antibiotic-sensitive staphylococci. When a single dose of kanamycin (25 mg/kg) was given 1 hr after injection of the blood-bacteria mixture, the abscesses were 100% sterilized with 8 negative and no positive cultures; an injection, 24 hr postinoculation, sterilized no abscesses and eight cultures were positive. When multiple doses of penicillin and kanamycin were administered for 7 days beginning immediately after the inoculation of the infected blood, the results were similar to those obtained with a single injection, but when multiple injections were begun 7 days after the inoculation of the infection the abscess was sterilized in only one out of seven animals (Table 3).

TABLE 3. *Effect of multiple doses of penicillin and kanamycin on cultures of abcesses with injections begun immediately and 7 days after start of infection*

| Antibiotic | Daily dosage | Time of initial injection | Sterilized abcesses | Number negative cultures | Number positive cultures |
|---|---|---|---|---|---|
| | | | % | | |
| Kanamycin | 25 mg/kg | Immed. | 100 | 8 | 0 |
| Kanamycin | 25 mg/kg | 7 days | 14 | 1 | 6 |
| Penicillin G | 7,000 units | Immed. | 100 | 4 | 0 |
| Penicillin G | 7,000 units | 7 days | 14 | 1 | 6 |

The effect of local instillation of antibiotics on the survival of persisters was studied by allowing the abscess to localize and injecting the antibiotic directly into the abscess cavity. When a single injection of penicillin was administered immediately after the injection of the infected blood, infection could be prevented, but when a single injection was given 7 days later the abscesses were not

sterilized (Table 4). However, when the animals were treated for 7 days with multiple injections of penicillin G and kanamycin, the abscesses were sterile when the animals were sacrificed a week later.

TABLE 4. *Effect of single local injection of penicillin G on cultures of abcesses 7 days after infection*

| Dosage | Sterilized | Number negative cultures | Number positive cultures |
|---|---|---|---|
| *units* | % | | |
| 5-10 | 33 | 1 | 2 |
| 30-100 | 0 | 0 | 3 |
| 800-1000 | 33 | 1 | 2 |
| 8000-10,000 | 0 | 0 | 3 |
| Control | 0 | 0 | 3 |

*Role of antibiotic-bacterial contact in vivo.* Since much has been written about anatomic barriers preventing diffusion of antibiotics and thus accounting for the survival of sensitive bacteria, a series of experiments was carried out to investigate further the role of antibiotic-bacterial contact as a factor in microbial persistence. These experiments were not designed to study the effect of anatomic barriers on antibiotic diffusion, but were planned so as to provide the greatest opportunity for diffusion to take place, to demonstrate the antibiotic in the cavity, and to study the effect of the presence of the antibiotic on the survival of bacteria. Infected hematomas were allowed to develop into well-localized abscesses, and a single large dose of penicillin was given intramuscularly. The rabbits were sacrificed at 1, 2, 3, 4, and 12 hr following the injection, the abscesses cultured, and the exudate studied for penicillin content. No attempt was made to measure precisely the level of the penicillin in the exudate but the zone of inhibition of a test organism *(Sarcina lutea)* produced by the exudate was compared to that produced by a solution of penicillin containing 1 unit per ml. The minimal inhibitory concentration of the infecting staphylococcus was 0.1 that of the standard solution (0.1 units/ml). In each instance the exudate produced as large or larger a zone than the standard penicillin solution,

TABLE 5. *Zone of inhibition of growth of Sarcina lutea produced by exudate of treated and untreated animals compared to standard penicillin solution*

| Inhibition | Standard penicillin (1 unit/ml) | Untreated animals | Treated animals |
|---|---|---|---|
| | *mm* | | *mm* |
| Average | 9 | 0 | 17 |
| Range | 8.6 to 9.4 | 0 | 8.8 to 36.0 |

indicating good penetration of the penicillin into the exudate, in concentrations in excess of 10 times the minimal inhibitory concentration of the infecting organism (Table 5). In spite of the presence of this amount of penicillin, antibiotic-sensitive bacteria could consistently be removed from the exudate. These results confirm the reports of others that antibiotic-bacterial contact in vivo does not prevent the persistence of sensitive bacteria.

Recently, experiments have been carried out utilizing the implantation of bacteria-containing cellophane bags in the peritoneal cavities of mice. These are incomplete at this time, but microbial persistence has been demonstrated with two of the newer 6-amino penicillanic acid derivatives, penicillin P-12 and SK&F 12141-Z.

## Literature Cited

Barclay, W. R., R. H. Ebert, G. V. Leroy, R. W. Manthei, and L. J. Roth. 1935. Distribution and excretion of radioactive isoniazid in tuberculous patients. J. Am. Med. Assoc. **151**: 1384-1388.

Bigger, J. W. 1944. The treatment of staphylococcal infections with penicillin by intermittent sterilization. Lancet **2**:497-500.

Canetti, G. 1955. The tubercle bacillus in the pulmonary lesion of men: histobacteriology and its bearing on the therapy of pulmonary tuberculosis. Springer Pub. Co., New York.

Eagle, H. 1952. An experimental approach to the problem of treatment failure with penicillin. I. Group A streptococcal infection in mice. Am. J. Med. **13**:389-399.

Hobby, G. I., L. Meyer, and E. Chaffee. 1942. Observations on mechanisms of action of penicillin. Proc. Soc. Exptl. Biol. Med. **50**:281-285.

McCune, R., S. H. Lee, K. Deuschie, and W. McDermott. 1957. Ineffectiveness of isoniazid in modifying the phenomenon of microbial persistence. Am. Rev. Tuberc. Pulmonary Diseases **76**:1106-1109.

McDermott, W. 1958. Microbial persistence. Yale J. Biol. Med. **30**:257-291.

Ramelkamp, C. H., and W. M. Kirby. 1945. Factors determining the dosage of penicillin in the treatment of infection. Bull. New York Acad. Med. **21**:656-672.

Smadel, J. E., R. Traub, R. L. Ley, Jr., C. B. Phillip, T. E. Woodward, and R. Lewthwaite. 1949. Chloramphenicol (Chloromycetin) in the chemoprophylaxis of scrub typhus (Tsutsugamushi Disease). II. Results with volunteers exposed in hyperendemic areas of scrub typhus. Am. J. Hyg. **50**:75-91.

# CORNEAL *PSEUDOMONAS* INFECTIONS IN EXPERIMENTAL ANIMALS AND MAN

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Corneal disease caused by strains of the genus *Pseudomonas* is a severe problem in ophthalmology. The organism is usually inoculated into the corneal layers with small metallic foreign bodies or is a contaminant in the solutions used at the time of foreign body removal. *Pseudomonas* finds the human corneal stroma to be a hospitable medium, and the resulting ulcer may become an unmanageable keratitis in 24 to 48 hr. Thus, an ophthalmologist who observes a central corneal ulcer of recent duration, subsequent to trauma, must consider the possibility of infection with *Pseudomonas*, especially since early management is essential. The continuous introduction of new antimicrobial agents which may be suitable for the treatment of corneal *Pseudomonas* infection has made imperative the development of techniques for testing these preparations.

The drug of choice in preventing and in treating this disease has yet to be determined. Bignell (1951) obtained encouraging results with daily subconjunctival injections of streptomycin. Penicillin and the sulfonamides have been used by earlier investigators (Brown, 1943; Joy, 1942; Juler and Young, 1945) with equivocal results. More recently, encouraging results have been obtained experimentally with polymyxin B and E by Ainslie and Smith (1952) and Wiggins (1952). Colistin (Colymycin) has been used successfully by Gordon and McLean (1960).

In vitro studies (Truant, 1961) of some 400 strains of *Pseudomonas* demonstrated that polymyxin B is superior to other available agents. Therefore, the experiments reported here were designed to test its in vivo efficacy and to compare it with other drugs.

## Materials and Methods

These experiments utilized 125 adult male white rabbits, weighing approximately 2 kg. One eye of each rabbit was infected and all rabbits in a given experiment were inoculated at one time. Corneal

inoculation was achieved with a technique utilizing the contamination of a needle track. This method involves the use of a curved Grieshaber 28/7 corneal needle (Ernest Grieshaber, Schaffhausen, Switzerland) threaded with 6-0 black silk that has been saturated with *Pseudomonas*. The needle is used to make a track through about half the corneal thickness. The contaminated thread is drawn through the needle track, thus depositing the inoculum in the corneal lamellae.

Eleven strains of *Pseudomonas* isolated from a variety of clinical sources (eye, blood, sputum, urine, wound, etc.) were used. All eye infections were produced with 18-hr cultures grown in Trypticase soy broth, washed three times, and resuspended in saline. In vitro sensitivity tests were performed on the isolates (Truant, 1961), using both tube dilution and disc-plate procedures. The 11 strains used throughout the experiments showed minimal inhibitory concentrations (MIC) which varied from 50 to 500 units per ml of polymyxin B (F. Fink, Chas. Pfizer and Co., Brooklyn, N.Y., kindly supplied the polymyxin B.) Strain no. 1 had an MIC of 100. Strains no. 6, 55, and 56 had MIC's of 50. We arbitrarily chose concentrations of 100 and 1000 units per ml of this drug in our treatment procedure.

Treatment of the inoculated eye was initiated within 2 to 3 hr after producing the needle track. A novel procedure was used to deliver the chemotherapeutic agent. This consisted of a subpalpebral drip of the antibiotic solution (100 or 1000 units per ml) at the rate of 0.5 ml per min. In addition to this constant therapeutic bath, a vigorous flushing of the eye with 0.85% saline was employed which automatically irrigated the eye at 2-hr intervals. The flushing procedure effectively removed the purulent exudate characteristic of *Pseudomonas* keratitis.

*Details of the delivery procedure.* The involved area is anesthetized with 2% lidocaine hydrochloride. A 5-gauge trochanter is inserted into the upper cul-de-sac in its posterior third. The trochanter is slipped over the edge of the bony upper orbital rim and into the subdermal area of the scalp. It is forced beneath the skin until the tip of the trochanter is felt to be at the base of the ear. There it is brought out through the scalp. A flexible nonkinking vinyl scalp-vein tubing, produced by Abbott Laboratories, is then slid into the trochanter far enough so that when the trochanter is pulled out from between the ears, the vinyl tubing is left with its flanged end under the upper lid and its free end emerging between the ears. (Mr. Lund of Abbott Laboratories, Hospital Department, Chicago, Ill., very kindly supplied the "scalp-vein tubing" and solvent for the apparatus.) By cutting off the heavy plastic cuff (designed to be slipped into a needle shank when used as a scalp-vein infusion set), a small flange is left attached to the vinyl tubing. We find this flange useful in keeping the tube in place under the lid. Where the vinyl tubing emerges from the skin, adhesive tape is wrapped about it to prevent

migration from under the lid. The distal end of this vinyl tube is joined, using a metal union and Abbott vinyl solvent, to a length of the same tubing which ultimately is attached to the source of the chemotherapeutic agent. This is accomplished by passing the tubing through an L-shaped metal antenna-like tube held in place on the rabbit's back by an adjustable canvas harness. The vinyl tubing emerges from the top of this antenna and is connected to a small swivel valve fixed in the roof of the animal cage. This valve is subsequently connected to a line leading into a two-way valve permitting the flow of chemotherapeutic agent as well as the intermittent delivery of saline. The antibiotic is delivered by gravity flow from a manifold system. The normal saline is pumped in by a small motor.

Once in operation, the system is automatic but it does require periodic surveillance to assure trouble-free function and to evaluate the status of the corneal infection. After each experiment, the lines are flushed with a 5% phenol solution and adequately rinsed with normal saline.

The animals may circle about the cage without twisting off the delivery tubing. They rarely detach the connections. This apparatus has proven far superior to the method of restraint utilizing small wooden stanchions (similar to those used by dairymen) which we employed in previous experiments. The rabbits frequently "thrashed about" so wildly in these stanchions that several fractured their spines and developed hemiplegia.

We used Wiggins' technique in grading corneal ulcers: 0 = no ulceration (0 mm); 1x = central infiltration, healed with faint scarring (1 to 2 mm); 2xx = dense central infiltration, healed with dense central scarring (2 to 4 mm); 3xxx = more than $\frac{1}{2}$ of cornea infiltrated, healed with dense scarring (4 to 10 mm); and 4xxxx = whole cornea infiltration, healed with completely vascularized scar, or cornea perforated. We feel that it may have some clinical significance in that grades 1x to 3x would probably be amenable to surgery but grade 4x probably would not.

## Results

The first six experiments leading to the development of the present technique of inoculation and treatment have been reported previously (Hessburg, Truant, and Penn, *in press*. Only the cumulative data of recent experiments with polymyxin B are detailed herein.

Treatment with polymyxin B prevented keratitis in 30% (9 of 30) animals; 50% (15 of 30) showed corneal damage of 1x to 3x. Although 20% (6 of 30) of the eyes represented therapeutic failure, it should be noted that four of these six animals were inadequately treated owing to failures in the delivery system. All control animals showed extensive disease (4x). Grading of damage was done at the

end of the treatment period. Animals in group 4x were killed after the treatment period. All other animals were observed at least 28 days for recurrences.

In experiment 7 (Table 1), a strain of *Pseudomonas* isolated from sputum was used. The animals were treated for an 8-day period, and keratitis was satisfactorily controlled in three animals. The control eye perforated and this animal was killed. Of interest in this experiment was the finding that cultural techniques demonstrated organisms after 7 days in all of the treated as well as the control eyes.

TABLE 1. *Incidence of Pseudomonas* corneal ulcers after 8 days of treatment with 100 units/ml of polymyxin B*

| Rabbit no. | Gradation of ulcer | Organisms present after 7 days |
|---|---|---|
| 1 | 2x | Few |
| 2 | 2x | Few |
| 3 | 1x | Few |
| 4 K | 4x | 4+ |

*Strain 1, sputum isolate.

Experiment 8 (Table 2) demonstrated an extremely interesting fact: three of four animals after a 4-day treatment period did not have clinically apparent corneal disease nor did they have positive cultures. When treatment was discontinued, however, corneal infection became apparent within 2 days.

TABLE 2. *Incidence of Pseudomonas* corneal ulcers after 4 days of treatment with 1000 units/ml of polymyxin B*

| Rabbit no. | Gradation of ulcer | | Organisms present after 4 days |
|---|---|---|---|
| | 4 days | 6 days | |
| 1 | 0 | 1x | None |
| 2 | 0 | 1x | None |
| 3 | 0 | 1x | None |
| 4 | 2x | 2x | 3+ |
| 5 K | 4x | 4x | 4+ |

*Strain 6, urine isolate.

In experiment 9 (Table 3), the animals did not show an exacerbation of keratitis when treatment was stopped after 8 days, even though there were organisms cultured from the cul-de-sac.

In experiments 10 and 11 (Tables 4 and 5), difficulties were encountered with the apparatus. Two of the animals in each experiment

developed severe keratitis which may not have become as severe if the delivery of agent had been constant.

TABLE 3. *Incidence of Pseudomonas* corneal ulcers after 8 days of treatment with 1000 units/ml of polymyxin B*

| Rabbit no. | Gradation of ulcer | Organisms present after 4 days |
|---|---|---|
| 1 | 1x | Few |
| 2 | 2x | 2+ |
| 3 | 0 | – |
| 4 | 1x | Few |
| 5 | 2x | Few |
| 6 | 0 | – |
| 7 K | 4x | Few |

*Strain 1, sputum isolate.

TABLE 4. *Incidence of Pseudomonas* corneal ulcers after 5 days of treatment with 1000 units/ml of polymyxin B*

| Rabbit no. | Gradation of ulcer | Organisms present after 4 days |
|---|---|---|
| 1 | 4x | Few |
| 2 | 4x ] Treatment | Few |
| 3 | 3x ] interrupted | 2+ |
| 4 | 0 | – |
| 5 | 0 | – |
| 6 K | 4x | 2+ |
| 7 K | 4x | 4+ |

*Strain 55, eye isolate.

TABLE 5. *Incidence of Pseudomonas* corneal ulcers after 7 days of treatment with 1000 units/ml of polymyxin B*

| Rabbit no. | Gradation of ulcer | Organisms present | |
|---|---|---|---|
| | | 4 days | 7 days |
| 1 | 2x | 5 col. | 3 col. |
| 2 | 4x ] Treatment | 2 col. | 4+ |
| 3 | 4x ] interrupted | 4+ | Few |
| 4 | 2x | Few | Few |
| 5 | 0 | Few | – |
| 6 | 4x | Few | 5 col. |
| 7 K | 4x | 4+ | 4+ |

*Strain 56, urine isolate.

Experiment 12 (Table 6), as well as data in several of the other experiments, aptly demonstrated the variability between cultural results and clinical results. Certainly no prognosis concerning exacerbations may be made with cultural findings only. Table 7 summarizes the findings of the six experiments with polymyxin B.

TABLE 6. *Incidence of Pseudomonas* corneal ulcers after 14 days treatment with 1000 units/ml of polymyxin B*

| Rabbit no. | Gradation of ulcer | Organisms present after 4 days |
|---|---|---|
| 1 | 2x | – |
| 2 | 3x | 5 col. |
| 3 | 3x | 5 col. |
| 4 | 0 | – |
| 5 | 2x | – |
| 6 | 2x | – |
| 7 K | 4x | 4+ |
| 8 K | 4x | 4+ |
| 9 K | 4x | 4+ |

*Strain 57, eye isolate.

TABLE 7. *Summary of experiments on Pseudomonas corneal ulcers*

| Expt no. | No. of rabbits | Days of treatment | Results of treatment | | | Controls | | Recurrences |
|---|---|---|---|---|---|---|---|---|
| | | | No. ulcer | 1x - 3x | 4x | No. of rabbits | 4x Ulcer | |
| 7 | 4 | 8 | 0 | 3 | 0 | 1 | 1 | 0 |
| 8 | 5 | 4 | 3 | 1 | 0 | 1 | 1 | 3 |
| 9 | 7 | 8 | 2 | 4 | 0 | 1 | 1 | 0 |
| 10 | 7 | 7 | 2 | 1 | 2 | 2 | 2 | 0 |
| 11 | 7 | 7 | 1 | 2 | 3 | 1 | 1 | 0 |
| 12 | 9 | 14 | 1 | 5 | 0 | 3 | 3 | 0 |
| Totals | 39 | | 9 | 16 | 5 | 9 | 9 | 3 |

## Discussion

In the past, difficulty has been encountered in producing corneal ulcers with *Pseudomonas* in rabbits. This has been attributed to variations in resistance among animals. This suspicion was initially confirmed by failure of several commonly used experimental methods involving scarification techniques. The first of these experiments used only simple inoculation of massive numbers of *Pseudomonas* into the cul-de-sac of 10 rabbits; no infections were produced. Abraiding the rabbit cornea plus cul-de-sac inoculation, without intentionally damaging Bowman's membrane or corneal stroma, yielded

two ulcers in seven attempts. Injury to the corneal epithelium with 5% phenol, followed by the instillation of massive quantities of inoculum, was unsuccessful in seven of seven rabbits. There were no ulcers produced in 14 guinea pigs after use of various scarification techniques. (Intralamellar injection is effective in producing ulcers but has objectionable features.) The present technique, utilizing the contamination of a needle track, has resulted in successful induction of keratitis in 50 consecutive untreated rabbits.

The needle track and contaminated thread procedure has several advantages. (i) The corneal epithelium is more nearly intact after inoculation. (ii) The small epithelial wound more closely mimics those seen in humans after foreign body removal. (iii) There is no difficulty in identifying keratitis at an early stage (as there is with intralamellar injection of inoculum), since any resulting grayish opacity is abnormal. Results may be evaluated with greater reliability when treatment is initiated early (2 to 3 hr after inoculation), rather than waiting for typical ulcers with wide epithelial destruction and extensive stromal involvement. (iv) The effects of tears, lids, and nictitating membrane are negated. (v) Organisms of lower invasiveness are given a more suitable opportunity to produce infection.

*Treatment of keratitis in experimental animals.* The problem of treating the keratitis which results from successful inoculation of *Pseudomonas* into corneal stroma has several interesting facets. The speed of progression and the copious amounts of purulent exudates produced are particularly important. Many investigators have used local drops or ointments administered at varying intervals. This method has proven cumbersome in our hands and makes it difficult to evaluate a significant number of animals. Furthermore, the local intermittent use of such medications affords only brief exposure of the ulcer to the agent.

There have been several changes in our more recent methods. These involve the treatment apparatus and are worthy of mention. Substitution of vinyl scalp-vein tubing for the polyethylene used originally has reduced failures in delivery of the agent. Vinyl tubing did not kink or crack as readily as polyethylene. When an occasional leak does occur, repair is simplified by use of the vinyl solvent. An occasional animal succeeded in pawing the tubes out when they were exteriorized at the upper lid. This can be prevented by tunneling the tubing beneath the scalp and bringing it out between the ears. We originally sutured the tubing in place with one silk suture through the upper lid. In several animals, this needle hole allowed chemotherapeutic agent to invade the tissues of the lid and scalp. By using a flanged tubing, no sutures need be placed through the tubing and this problem was eliminated.

The flush system uses normal saline under pressure every 2 hr, and rids the eye of excessive purulent material, preventing the blockage of the delivery tube with purulent material. This is considered

important in experiments with *Pseudomonas*. Maximal benefits from this flush are obtained in the first 2 min of flow; a longer period than this is dangerous, since the animal may inspire highly infectious fluids. This embarrasses the pulmonary reserve; in two instances, we suspect that animals were lost to *Pseudomonas* pneumonia. The harness utilizes Velcro (American Velcro Corp., Manchester, N.H.), which is easier to apply and remove than metal buckles.

The data on treatment illustrate several points which may be of importance. The development of ulcers after 4 days of continuous treatment and subsequent discontinuance of treatment shows that this organism is able to lie dormant for some time within tissues. This would suggest that drugs need be used for a considerable period of time to insure against exacerbation.

The ability of this organism to create infections while under massive bombardment with polymyxin B is amazing. Nine animals receiving 720,000 units per day of polymyxin B in a corneal bath developed infections. It is true that the severity of these infections was ameliorated; nonetheless these animals illustrate the remarkable suitability of the cornea as a medium for this organism. The failure of local therapy using strengths of polymyxin B now available commercially may be partially explained on this basis.

We hasten to state that we do not feel these experiments are immediately transferable from animal to man. They do, however, suggest the possibility of some changes in clinical management of these infections.

*Studies on human corneal infections.* We recently treated *Pseudomonas* keratitis in four humans. The most recent of these was managed with local polymyxin B alone; the other three were given additional subconjunctival injections of polymyxin B. We used a solution containing 2,200 units of polymyxin B per drop. Two drops of this were instilled every 30 min around the clock. The eye was thus exposed to more than 200,000 units locally each day. Subconjunctival injections of 25,000 to 50,000 units of polymyxin B were given for 5 to 10 days. The local drops were continued for at least 2 weeks, with a gradual increase in the time interval between drops.

The case managed locally and two of the other three cases had satisfactory visual results. The case which failed was extensively involved when first seen, and, though controlled by polymyxin B, irrevocable damage had been done and evisceration was subsequently necessary to control pain. Details of the management of these cases will be presented at a later date.

## Literature Cited

Ainslie, D., and C. Smith. 1952. Polymyxin E penetration into the eye and therapeutic value in experimental infection due to *Pseudomonas pyocyaneus*. Brit. J. Ophthalmol. **36**:352-361.

Bignell, J. L. 1951. Infection of the cornea with *B. pyocyaneus*: clinical study and summary of ten cases personally observed. Brit. J. Ophthalmol. **35**:419-423.

Brown, E. H. 1943. Therapeutic experiences with corneal ulcer due to *Bacillus pyocyaneus*. Arch. Ophthalmol. **30**:221-224.

Gordon, D. J., and J. M. McLean. 1960. Colistin in *Pseudomonas* infection. Am. J. Ophthalmol. **50**:33-35.

Joy, H. H. 1942. Treatment of experimental *Bacillus pyocyaneus* ulcer of cornea with sulfapyridine. Arch. Ophthalmol. **27**:1135-1164.

Juler, F., and M. Y. Young. 1945. The treatment of septic ulcer of the cornea by local applications of penicillin. Brit. J. Ophthalmol. **29**:312-322.

Truant, J. P. 1961. In vitro evaluation of the commonly used chemotherapeutic agents against isolates. Antimicrobial Agents Ann. 1960, p. 145-151.

Wiggins, R. L. 1952. Experimental studies on the eye with polymyxin B. Am. J. Ophthalmol. **35**:83-99.

# EPIDEMIOLOGY OF URINARY TRACT INFECTIONS IN A POPULATION OF "NORMAL" FAMILIES

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Recent epidemiological studies by a number of investigators, including ourselves, have yielded valuable information on the prevalence of urinary tract infections in various population groups. The present study was designed to learn whether multiple cases of significant bacteriuria occur within families and, also, whether there is a seasonal variation in the incidence of such infections. The study is still in progress but the preliminary findings are presented here.

## Materials and Methods

A number of young families and a smaller group of "older" families are under our continuing study, primarily to determine the prevalence and intrafamilial spread of respiratory infections. Clean-voided urine specimens from this ambulant "normal" population were obtained in the winter, spring, and summer seasons of 1961, to determine the seasonal and familial frequency of urine abnormalities, both by culture and microscopy.

Each family (mostly Negro) is seen every two weeks by a staff member to determine and record the presence of respiratory or other illnesses. Throat swabs for bacterial and viral studies are obtained at each visit. During the past three seasons, a midstream voided-urine specimen was collected in a sterile, plastic, 135-ml container from almost all participants. This was either refrigerated or brought to the laboratory within one-half hour. All specimens were examined microscopically and cultured within a few hours after arrival in the laboratory. The specimens obtained in the winter season had their bacteria enumerated by the tube-dilution method. Subsequent specimens in the spring and summer were screened, as has been reported previously, by streaking a 3-mm loopful of urine onto nutrient agar. If bacterial growth occurred on the plate, tube-dilution titrations were performed.

Each specimen was screened for the presence of albumin and glucose with the paper-strip method. Repeat specimens were obtained whenever abnormal findings were detected.

The number of participating families declined from 46 to 29 families during the seasonal samplings because of family relocations, noncooperation, or waning interest in the over-all study. A somewhat larger proportion of the participants were females. The number of specimens collected ranged from 236 in the winter to 149 in the summer.

## Results

In all three seasons, the proportion with bacterial counts of $10^5$ cells per ml or more was consistently larger in females, ranging from 15% in the winter to 29% in the spring, and 19% in the summer. Seasonally, significant bacteriuria varied from 10% in the winter to 19% in the spring, and 16% in the summer for the entire population. Significantly more males (12%) had bacteriuria in the summer, but the reason for this is obscure.

In almost every age group, a greater proportion of women was found to have significant bacteriuria. The proportion of "positives" was especially high in women 30 years old or older, varying from 20% in the summer to 34% in the winter, and 41% in the spring.

The occurrence of multiple cases of bacteriuria in family units was an unexpected finding in this study. Persistent "significant" bacteriuria in the mother and one or more children was observed in five families. In no instance was the father involved.

In one family, the mother was consistently infected but had a history of previous urinary tract infection. During the summer survey, her 5-year-old male child also had persistent *Escherichia coli* without symptoms or signs of infection.

The mother of another family had a persistent *Proteus vulgaris* infection accompanied by symptoms during the winter, but not in the other seasons. She, too, had a history of prior urinary tract infection. Her 2-year-old daughter had a persistent *E. coli* bacteriuria with no symptoms or signs of infection in the winter, but not in the later surveys. It is possible that the mother also had the same organism, but that it was masked by the *Proteus*.

In a third family, significant bacteriuria occurred in both the winter and summer seasons, with mild symptoms in the mother but not in her two children. *E. coli* was found in all three during the winter, *P. vulgaris* in two, and *Staphylococcus aureus* in the third child (7 years old) in the summer.

Only 5-year-old twins and their 3-year-old sibling in a fourth family had persistent bacteriuria. *P. vulgaris* was isolated in all

instances but *E. coli* and *Aerobacter aerogenes* were found in a repeat specimen from the 3-year-old girl. None of these children had either a history or evidence of urinary tract disease. Unfortunately, in this family, studies were possible only during the winter season.

In the fifth family, persistent bacteriuria was present in two members, the mother and her youngest child, a 2-year-old girl. Neither had a history of previous urinary tract infection. In all instances, *E. coli* was isolated.

Persistent bacteriuria, defined as 100,000 or more bacteria/ml in the initial specimen and at least one repeat specimen in the same season, was found in 20 persons (9%) in the winter, 10 (5%) in the spring, and 7 (5%) in the summer. More than half (54%) of all cases of persistent bacteriuria occurred in adult women.

There were 11 pregnant women in the family population during this study period. Two were seen only in their antepartum periods, four, only postpartum, and five in both. Persistent bacteriuria was present in two of seven patients cultured antepartum, and in three of the nine who were postpartum. All of these women were multiparous, with three to 10 pregnancies. There seemed to be no positive correlation between the number of pregnancies and the persistence of bacteriuria. However, of the five patients, one had had nine and two others, 10 pregnancies. There was, also, no positive correlation between persistent bacteriuria and the presence of white blood cells in the urine.

Albuminuria consistently occurred more often in women, and in the winter and spring. The occurrence of white blood cells was about twice as frequent in the female in all three seasons but showed a marked decrease in both men and women in the summer. Red blood cells were seen only rarely. In the females this generally was almost, but not always, related to the menstrual periods of the patients. Glucosuria was noted on two occasions in patients known to have diabetes mellitus.

## Discussion

Our findings thus far serve to corroborate the earlier reports of Kass, Miall, and Stuart (1961), Kunin, Southall, and Paquin (1960), and others, that bacteriuria is more frequent in girls and women, especially in the women over age 30.

Because this was designed as a study of a family population, all of the adult females have had children. Although the numbers are small, it appears that persistent bacteriuria is common in the ante- and postpartum periods and that this may smolder along with few or no symptoms of urinary tract infection in multiparous women.

The finding of multiple instances of persistent bacteriuria within families is of considerable interest, particularly since organisms of

the same genus were present in four of the five families. This is to be pursued in another, larger group of families who are currently under study.

It is too early to be certain that there are significant differences in the seasonal occurrence of bacteriuria, with the winter season highest, but this, too, is to be investigated further.

## Acknowledgments

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## Literature Cited

Kass, E. H., W. E. Miall, and K. L. Stuart. 1961. Relationship of bacteriuria to hypertension: an epidemiological study. Abstr. Am. Soc. for Clin. Invest., p. 36.

Kunin, C. M., I. Southall, and A. J. Paquin. 1960. Epidemiology of urinary tract infections: a pilot study of 3,057 school children. New Engl. J. Med. **263**:817-823.

# LABORATORY AND CLINICAL STUDIES OF *ESCHERICHIA COLI* INFECTIONS

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*Escherichia coli* species are the most common offenders in acute, chronic, and asymptomatic infections of the urinary tract. Although clinical and epidemiologic studies have demonstrated that certain specific *E. coli* serotypes are more frequently incriminated in diarrheal disease of the infant and newborn, it has not been ascertained whether certain strains are also more commonly associated with urinary tract infections. The concept that some strains of gram-negative organisms may be associated with infection more often than others is not new. However, evidence for this idea rests mostly on inferential data derived from the study of infantile diarrhea or urinary tract infections with *Klebsiella* (Ørskov, 1952, 1954) and *Pseudomonas* on urologic wards (McLeod, 1958).

Vahlne (1945) and Sjöstedt (1946) performed serologic typing on strains of *E. coli* from a variety of normal and abnormal sources and found that certain types were more commonly associated with human infections. However, these types were not limited to the urinary tract. Kauffmann *(cited by* Sjöstedt, 1946) advanced the hypothesis that certain coliform serotypes are more prevalent in appendicitis. Many early investigators, attempting to implicate strains of *E. coli* with specific disease processes, confined their investigations to the use of biochemical tests for differentiation of these organisms. Demonstration and refinement of serologic techniques to identify *E. coli* has led to renewed interest in the relationship of serologic types to pathogenicity.

The purpose of this investigation is to assess whether certain strains of *E. coli* are more prevalent in bacteriuria than in other pathologic processes and to correlate cultural characteristics, biochemical reactions, antibiotic sensitivity patterns, and clinical features of the infection with serologic groups of *E. coli*.

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## Materials and Methods

*Organisms.* A total of 219 consecutive strains of indol-positive, lactose-positive, gram-negative bacilli, isolated from patients with coliform infections hospitalizated at the King County Hospital, was studied. These strains were presumed to be *E. coli* species on initial report by the hospital diagnostic bacteriology laboratory. This communication deals primarily with 100 urinary and 50 nonurinary isolates found to be biochemically characteristic of *E. coli* and serologically typable. Of the remaining strains, 41 urinary and 13 nonurinary isolates conformed to the biochemical patterns of *E. coli* but were not typable with available typing sera; and 15 isolates proved to be probable indol-positive *Klebsiella-Aerobacter* species.

*Biochemical tests.* A modified scheme of Edwards and Ewing (1955) was used for group differentiation of Enterobacteriaceae by biochemical tests; this included fermentation studies with nine different sugars and reactions with three amino acids. In addition, indol, methyl red, Voges-Proskauer, Simmon's citrate, triple sugar iron agar, and motility studies were performed on all isolates.

*Serology.* Typing sera were made available by the Communicable Disease Center Diagnostic Reagents Laboratory, U.S. Public Health Service. 0 antigen grouping was performed on all isolates showing a typical biochemical pattern of *E. coli.* Complete serological typing of *E. coli* cultures is dependent upon determination of the 0 group followed by characterization of K and H antigens. This study is limited to determination of the 0 (somatic) antigen, and although the term "serotyping" is frequently used, sero-grouping would be preferable. The scheme used for determinations of 0 antigen has been published in detail by Edwards and Ewing (1955).

*Antibiotic sensitivities.* Typable strains of *E. coli* were tested against penicillin, tetracycline, chloramphenicol, streptomycin, polymyxin B, and kanamycin, using a serial tube-dilution technique and measuring bacteriostatic end points. A $10^{-4}$ inoculum of an 18-hr broth culture was used.

*Clinical.* After determination of the 0 group, the clinical records of the 100 patients with *E. coli* bacteriuria were reviewed, with particular emphasis on symptoms of urinary tract infection, chronicity, pregnancy, catheterization, and instrumentation. All patients had significant bacteriuria, i.e., bacterial counts equal to or greater than 100,000 organisms per ml of urine. To facilitate analysis, data were assembled on marginal punch cards.

## Results

*Serology.* The prevalence of antigenic 0 groups among urinary and nonurinary isolates is shown in Table 1. There was no significant

difference in the distribution of the somatic antigens between urinary and nonurinary isolates except that 0 group 50 was limited to strains cultured from urine. Subsequently, organisms belonging to 0 group 50 have been found in extra-urinary foci. Strains containing 0 antigens (6, 75, 4, and 50) accounted for 62% of typable urinary cultures, and types 6, 75, and 4 comprised 52% of the strains cultured from extra-urinary sources. 0 group 6 was the most common somatic antigen, characterizing *E. coli* obtained from urine and other sources, and accounted for 26% of typable strains. Inter- and intra-cross reactions between 0 groups were encountered infrequently.

TABLE 1. *Prevalence of O groups among E. coli from urinary and extra-urinary sources*

| O group | Urine | Non-urine | Total |
|---|---|---|---|
| | *no.* | *no.* | |
| 6 | 26 | 13 | 39 |
| 75 | 18 | 9 | 27 |
| 4 | 10 | 4 | 14 |
| 50 | 8 | 0 | 8 |
| Miscellaneous | 38* | 24† | 62 |
| Nontypable | 41 | 13 | 54 |
| Total | 141 | 63 | 204 |

*38 strains distributed among 23 O groups
†24 strains distributed among 17 O groups

Nontypable strains were found with approximately the same frequency among urinary and nonurinary isolates; the majority of these were rough variants and could not be typed due to their particulate characteristics. Rough strains were repeatedly plated on blood agar in an attempt to produce a smooth form; this was not successful. In all, 71% of urinary and 79% of nonurinary isolates contained somatic antigens which could be characterized with available typing sera.

*Biochemical tests.* Fermentation of sucrose was the only test showing some relationship with serologic grouping, and all 0-75 isolates fermented sucrose, whereas none of the 0-4 or 0-50 groups fermented this carbohydrate. There were no differences in the chemical reactions among urinary and nonurinary isolates, and organisms from acute, symptomatic urinary tract infections did not differ biochemically from those cultured from patients with chronic, asymptomatic bacteruria (Table 2). This contrasts with the observations of Jackson (1960) who found that only 30 to 40% of *E. coli*, recovered during initial bouts of urinary infection, fermented salicin or sucrose, and these reactions were positive in almost all *E. coli* cultured from patients with chronic, recurrent infection.

With few exceptions, these results agree with the findings of other investigators who have repeatedly shown that cultural characteristics

and biochemical tests correlate poorly with serologic groups of *E. coli*. However, these biochemical tests are necessary for differentiating various members of the Enterobacteriaceae from one another. In addition, many strains termed "paracolon" by virtue of late lactose fermentation and other intermediate biochemical reactions have common somatic antigens and are most likely *E. coli*.

*Antibiotic sensitivities.* Figure 1 compares the antibiotic sensitivity patterns of all strains with those of the most prevalent serologic groups. There was no correlation between 0 group and antibiotic sensitivity, and sensitive, as well as resistant, organisms were found within the same 0 group. It should be noted, however, that the *E. coli* isolates, resistant to 25 μg per ml of kanamycin, were all of 0 group 75. Over 65% of the strains were sensitive to 50 units of penicillin per ml and 20 to 35% were resistant to tetracycline, chloramphenicol, and streptomycin at blood levels which are usually attained in vivo. There was no difference in the pattern of antimicrobial sensitivity among *E. coli* cultured from urine and strains isolated from extra-urinary foci.

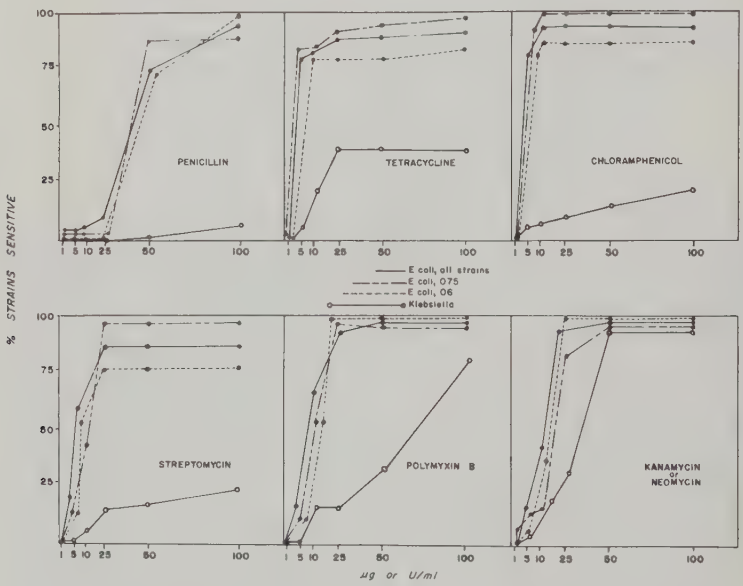


FIG. 1. *Correlation of serologic groups of E. coli with antimicrobial sensitivities.*

In numerous studies, *Klebsiella* have been included with *E. coli* under the common heading "coli-aerogenes." However, these organisms were consistently more resistant to antibiotics than *E. coli* (Fig. 1). Precise biochemical differentiation between these two genera

TABLE 2. *Comparison of O group and biochemical reactions*

| O Groups | Strains | Percentage of isolates with positive reactions | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | Glucose | Lactose | Sucrose | Mannitol | Salicin | Inositol | Adonitol | Sorbitol | Dulcitol | Indol | MR | VP | Citrate | Urea |
| | *no.* | | | | | | | | | | | | | | |
| 6 | 39 | 100 | 100 | 23 | 100 | 95 | 0 | 0 | 100 | 92 | 100 | 100 | 0 | 0 | 0 |
| 75 | 27 | 100 | 100 | 100 | 100 | 90 | 0 | 0 | 100 | 93 | 100 | 100 | 0 | 0 | 0 |
| 4 | 14 | 100 | 100 | 0 | 100 | 100 | 0 | 0 | 100 | 86 | 100 | 100 | 0 | 0 | 0 |
| 50 | 8 | 100 | 100 | 0 | 100 | 75 | 0 | 0 | 100 | 88 | 100 | 100 | 0 | 0 | 0 |

TABLE 3. *Clinical data on 100 patients with significant E. coli urinary tract infection*

| O Group | Strains | Symptomatic | Asymptomatic | Instrumented | Pregnant |
|---|---|---|---|---|---|
| | *no.* | *no.* | *no.* | *no.* | *no.* |
| 6 | 26 | 14 | 12 | 12 | 5 |
| 75 | 18 | 7 | 11 | 6 | 8 |
| 4 | 10 | 6 | 4 | 0 | 1 |
| 50 | 8 | 3 | 5 | 2 | 0 |
| Miscellaneous* | 38 | 21 | 17 | 4 | 0 |
| Total | 100 | 51 | 49 | 24 | 14 |

*38 strains distributed among 23 O groups.

is therefore necessary, not only from the point of view of taxonomy, but also because of its obvious therapeutic implications.

*Correlation of clinical data with serologic groups.* There was no appreciable difference in the somatic antigens of strains isolated from patients with symptomatic and asymptomatic infections of the urinary tract. Organisms with the same groups responsible for the majority of infections were also most prevalent in bacteriuria associated with instrumentation, chronic infection, and pregnancy (Table 3). No particular group was predominant among strains isolated from patients with severe and protracted infections. Of interest is a case which demonstrates the value of serotyping in studying the pathogenesis and progression of infection (Fig. 2). A 53-year-old man developed a symptomatic urinary tract infection following catheterization of the bladder. Subsequently, he had chills and fever, and *E. coli* was cultured from the blood on two occasions 12 days apart. He continued to have a low-grade fever despite antibiotic therapy. He was found to have a pulmonary infiltrate which progressed to cavitation, 10 days after the initial bacteremia. *E. coli* was the only organism isolated in significant numbers from numerous sputum, bronchial washing, and tracheal cultures. Repeated serologic typing demonstrated that a single pathogenic strain of *E. coli*, O-75, which was cultured from urine, blood, sputum, and trachea, was responsible for this illness. The

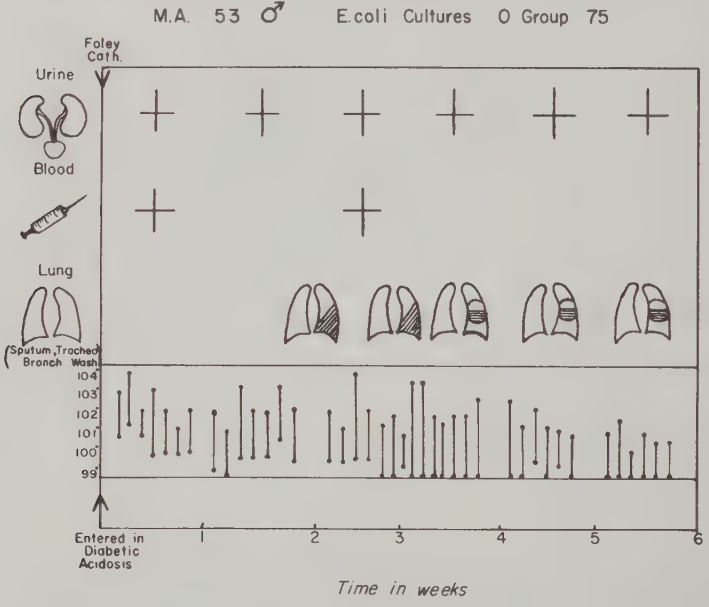


FIG. 2. *Course of a 53-year-old man with urinary tract infection, bacteremia, and lung abscess caused by a single strain of E. coli.*

organism acquired resistance to tetracycline while the patient was receiving treatment with this drug.

## Discussion

Extensive epidemiologic, clinical, and bacteriologic observations have documented the pathogenic significance of *E. coli* in infantile diarrhea. It seemed conceivable, therefore, that a few strains may also be responsible for the majority of urinary tract infections. Very little is known about the role of type-specific coliform bacteria in infections of the urinary tract. Ewing (1956), among others, has emphasized that complete serologic typing of *E. coli* should provide accurate information concerning the incidence of specific strains associated with disease and permit evaluation of nosocomial spread.

It is generally believed that the pathogens affecting the urinary tract emanate from the gut. Rantz *(unpublished data)* failed to find a relationship between 0 groups of *E. coli* found in the gut and in the urine of patients with infections; however, simultaneous urine and stool specimens from the same patient with urinary tract infection were not obtained. Preliminary observations in our laboratory indicate that untreated patients, with *E. coli* bacteriuria, have predominately the same serologic groups in the stool as in the urine, when multiple colonies from the stool are identified.

0 groups 6 and 75 accounted for the majority of urinary tract infections in Rantz's study, an observation that was corroborated by our work. We have, subsequently, performed sero grouping on many strains of *E. coli* from various hospitals in the Seattle area and from a teaching hospital in Baltimore. *E. coli* of 0 groups 6, 75, 4, and 50 have consistently been found in the majority of isolates, both from urinary and extra-urinary sources. Although preliminary, the available data support the concept that the majority of human *E. coli* infections are caused by a few specific antigenic groups. It does not seem likely, however, that specific strains have a marked predilection for renal tissue.

Although many bacterial genera have been incriminated in the pathogenesis of urinary tract infection, it is not clear how various bacterial species are related to one another. In particular, the commonly observed alteration in urinary flora, from *E. coli* to *Klebsiella, Proteus,* and *Pseudomonas* in patients with chronic infections following instrumentation and exposure to antimicrobials, remains an enigma. This may only represent selection of resistant strains following exposure to antibiotics; however, a change in flora may also occur in untreated patients. Beeson (1955) has emphasized that "clinicians have experienced the frustration of watching an infection due to one microbe subside, only to be superseded by an infection with another." With careful biochemical tests and serotyping to identify the etiologic pathogen in patients with chronic bacteriuria, it may

be established more clearly whether recrudescences are due to a new strain or represent re-infection with the same microbe. With this technique, we have been able to document that in some instances, at least, a group-specific strain acquires resistance to an antibiotic during therapy. More often, however, the initially sensitive strain appears to be replaced by a more resistant one of another bacterial genus. With the expansion of serologic techniques to include other gram-negative bacteria, more complete studies of this type will become possible. Aside from its epidemiologic value in tracing nosocomial infections, grouping of *E. coli* may provide further insight into the complicated host-parasite relationship existing in infections of the urinary tract.

## Acknowledgments

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## Literature Cited

Beeson, P. B. 1955. Factors in the pathogenesis of pyelonephritis. Yale J. Biol. Med. **28**:81-104.

Edwards, P. R., and W. H. Ewing. 1955. Identification of Enterobacteriaceae. Burgess Publishing Co., Minneapolis.

Ewing, W. H. 1956. Enteropathogenic Escherichia coli serotypes. Ann. N. Y. Acad Sci. **66**:51-70.

Jackson, G. G. 1960. Discussion, p. 479-480. *In* E. H. Kass and E. L. Quinn,[ed.], Biology of pyelonephritis. Little, Brown and Co., Boston.

McLeod, J. W. 1958. The hospital urine bottle and bedpan as reservoirs of infection by *Pseudomonas pyocyanea*. Lancet **1**:394-397.

Ørskov, I. 1952. Nosocomial infections with Klebsiella in lesions of the urinary tract. I. Acta Pathol. Microbiol. Scand. Suppl. **93**:259-271.

Ørskov, I. 1954. Nosocomial infections with Klebsiella in lesions of the urinary tract. II. Acta Pathol. Microbiol. Scand. **35**:194-204.

Sjöstedt, S. 1946. Pathogenicity of certain serological types of B. coli. Acta Pathol. Microbiol. Scand. Suppl. **63**:1-148.

Vahlne, G. 1945. Serologic typing of the colon bacteria. Acta Pathol. Microbiol. Scand. Suppl. **62**:1-127.

# STAPHYLOCOCCAL ENDOCARDITIS IN HEROIN ADDICTS

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Bacterial endocarditis is a frequent complication of heroin addiction, especially in addicts taking the drug by the "main line" or intravenous route. Hussey and Katz (1950) found an incidence of 8% in 102 heroin addicts with a variety of infections. Of the eight with endocarditis, seven had a staphylococcus as the causative organism; of these, four died. Autopsy revealed endocarditis limited to the tricuspid valve. These patients were characterized clinically by a triad of heroin addiction, septicemia, and pulmonary infarction without evidence of peripheral venous disease (Hussey et al., 1944; Wilhelm et al., 1947).

The present report concerns six heroin addicts with endocarditis seen on our service in the past 3 years. In addition to the patients of Hussey and Katz, two other patients with this condition have been reported (Holmes and Romansky, 1957; Wilder, 1957).

## Case Reports

Table 1 summarizes the clinical findings in the six patients.

Patient 1 presented with staphylococcal right lower lobe pneumonia which shortly progressed to bronchopleural fistula. Antibiotic therapy and surgical drainage led to prompt and complete clearing. A month after discharge he was readmitted with massive left empyema due to *Staphylococcus aureus*. Ristocetin was immediately begun, but several days after admission he developed a tricuspid systolic murmur, distended pulsating neck veins, and pulsation of the liver. Chest X rays showed numerous oval densities which later progressed to cavitation. The cardiac findings disappeared in about 1 week. The response to ristocetin was satisfactory, but convalescence was prolonged by an episode of embolization to the retina and by the appearance of several deep soft-tissue abscesses. No evidence of

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TABLE 1. *Staphylococcal tricuspid endocarditis in heroin addicts**

| Patient | Age, Sex | Chest X ray | Admission cultures | Heart murmur | Therapy | Remarks |
|---|---|---|---|---|---|---|
| 1 | 42 M | 1st Adm: Broncho-pleural fistula 2nd Adm. Septic pulm. infarction | *S. aureus*, blood, sputum, and pleural fluid | T. I. (Transient) | R 42 days | Response to ristocetin satisfactory but convalescence prolonged by retinal embolus and several deep soft-tissue abscesses (embolic ?) |
| 2 | 39 | Numerous oval densities in both lungs, progressing to cavitation | *S. aureus*, blood, sputum, and urine | Murmur of T.I., grew progressively louder | V 69 days | Blood cultures positive until 34th day. Patient readmitted 1 month after leaving hospital with heart failure and positive blood cultures. Lost to follow-up |
| 3 | 49 M | Several discrete oval densities in each lung field | *S. aureus*, blood | T.I. (transient) | P 21 days | Good clinical response. Lost to follow-up |
| 4 | 39 M | Numerous nodular densities in both lungs. Left pleural effusion | *S. aureus*, blood | Apical, systolic | V 3 days | Critically ill on admission. Oliguric throughout hospital course. Died 3 days after admission |
| 5 | 40 M | Several peripheral densities suggesting pulmonary emboli | Blood: neg. Sputum: neg. Skin abscesses: *S. aureus* Urine: *S. aureus* | none | V 21 days | Comatose on admission. Severe tachypnoea and resp. alkalosis for 3 days. Good response to therapy, but lost to follow-up |
| 6 | 31 F | Bilateral pleural effusions, confluent infiltrates suggesting pulmonary infarction | Blood: neg. Sputum: *S. aureus*. Urine: *S. aureus* | none | P 3 days C, E 21 days | Good response to therapy: *E. coli* pyelonephritis developed during convalescence; present on follow-up |

*Abbreviations used: C, chloramphenicol; E. erythromycin; P, penicillin; V, vancomycin; R, ristocetin; T.I., tricuspid insufficiency.

renal involvement was noted on this admission, but 1 year later he was found to have *Escherichia coli* pyelonephritis.

Patient 2 was hospitalized for symptoms of narcotic withdrawal. Physical examination revealed retinal hemorrhages, rales in the chest, and hepatomegaly. Chest X ray revealed several oval densities in each lung field. Several days later a tricuspid systolic murmur and peripheral evidence of tricuspid insufficiency were noted; these findings persisted throughout hospitalization. Blood cultures remained positive for *S. aureus* until the 24th day, despite vancomycin in adequate dosage. Convalescence was marked by several episodes of hematuria or pyuria or both; urine cultures yielded *S. aureus*. He slowly improved, but left the hospital against advice shortly after 69 days of therapy were completed. Four weeks later he was readmitted with right heart failure. Blood cultures were positive for *S. aureus*. Pyuria and positive urine cultures for *Pseudomonas* were noted during this admission. He again left the hospital against advice while blood cultures were positive, and has been lost to follow-up.

Patient 3 presented with a history of fever and hemoptysis for 1 week. Chest X ray was compatible with septic pulmonary embolization, and blood cultures yielded *S. aureus*. Penicillin produced a prompt clinical response, but convalescence was complicated by an episode of flank pain and hematuria, suggesting renal embolus. A tricuspid systolic murmur was heard transiently.

Patient 4 had chills, fever, and a cough productive of greenish sputum for 1 week prior to admission. Examination revealed a critically ill man with rales in both lungs and a soft apical systolic

TABLE 2. *Complications in staphylococcal tricuspid endocarditis in heroin addicts.*

| Patient | Peripheral emboli | Renal involvement | | Cardiac |
|---|---|---|---|---|
| | | Acute | Chronic | |
| 1 | Eye, arm, leg, and foot | None detected | *E. coli* pyelonephritis 1 year later | None |
| 2 | Eye, kidney | Recurrent episodes of hematuria and pyuria. Urine culture: *S. aureus* | Pyuria. Urine culture: *Pseudomonas* | Chronic right heart failure |
| 3 | Kidney | Episode of fever, red and white blood cells in urine during therapy | No follow-up | None at discharge. No follow-up |
| 4 | Kidney | Bun 97 oliguria | Died. Autopsy: numerous micro abscesses | Died. Autopsy: tricuspid endocarditis |
| 5 | Kidney | Fever, flank pain, red cells in urine 8th day. Urine cultures: *S. aureus* | No follow-up | None at discharge |
| 6 | Kidney | Fever 9th day, urine culture then positive for *S. aureus* and *E. coli* | Continued pyuria. Cultures: *E. coli* | None at discharge |

murmur. Chest X ray revealed bilateral nodular densities in the lungs, and left pleural effusion. Blood cultures grew *S. aureus*. The patient died after 3 days of vancomycin therapy at reduced dosage (because of oliguria). Autopsy revealed a large vegetation on the septal leaflet of the tricuspid valve, numerous septic pulmonary emboli, and multiple micro abscesses in the kidneys.

As noted, two of our patients have been designated as having probable tricuspid endocarditis. Both had septic pulmonary infarction, but positive blood cultures were not obtained. The staphylococcal etiology has been inferred from positive sputum and urine cultures. Both had an episode suggesting renal embolization during the hospital course, and patient 6 developed a gram-negative urinary tract infection (Table 2).

## Discussion

The clinical picture of staphylococcal tricuspid endocarditis in heroin addicts does not differ significantly from that seen in nonaddicts. The clinical findings are largely due to septic pulmonary embolism, and the manifestations of cardiac involvement may be minimal or absent. In one series of patients with autopsy-proved tricuspid endocarditis who were not addicts, only 3 of 15 had tricuspid murmurs (Bain et al., 1958), and tricuspid murmurs were heard in 8 of 20 autopsied cases in another (Goldburgh, Baer, and Lieber, 1942). Tricuspid systolic murmurs were heard in three of our patients, although in two they were transitory. The patient in whom tricuspid endocarditis was proved at autopsy had only a soft apical systolic murmur. By contrast, symptoms referable to pulmonary embolism were prominent in every patient, and were the dominant features of the clinical course in patients 1, 4, and 5.

Because the signs of pulmonary embolism are usually so prominent and evidence of cardiac involvement minimal or absent, the radiological findings of septic pulmonary infarction may be of cardinal importance in suggesting the diagnosis. The pulmonary lesions are usually multiple, and may appear first in one portion of the lung, then another. Serial films reveal central excavation and finally thin-walled cavities which usually close (Hussey and Katz, 1945).

In contrast to the previously reported cases, arterial embolism was noted in every patient in our series. As shown in Table 2, patients 1 and 2 had retinal emboli, and patients 2-6 had evidence of renal embolization. Pulmonary suppuration had been present for some time prior to these episodes in every instance, suggesting that the emboli may have originated in the lung rather than in the tricuspid valve itself. Bain et al. (1958) noted the same sequence of events, and suggested that these emboli arise from septic thrombophlebitis of pulmonary veins in the vicinity of abscesses.

Autopsy in patient 4 afforded an opportunity for pathological studies of the renal lesions. The numerous micro abscesses seen in this patient's kidneys were similar to ones we have seen in autopsy and renal biopsy specimens from other patients with staphylococcal endocarditis involving the valves of either side of the heart. Preliminary studies (in conjunction with A. E. Parrish, The George Washington University School of Medicine) employing percutaneous renal biopsy indicate that they may be as difficult as the valve lesions to eradicate, and that perhaps this technique may be of value in determining the duration of treatment. There is experimental evidence suggesting that these lesions may predispose to the subsequent development of pyelonephritis due to gram-negative bacilli. The kidneys of rats which have recovered from hematogenous staphylococcal pyelonephritis are quite susceptible to spontaneous infections by gram-negative bacilli (De Navaques, 1956). Patient 2 apparently developed *Pseudomonas* pyelonephritis as a sequel to his staphylococcal infection. The evidence for such a relationship is less clear in patient 1, who was found to have *E. coli* pyelonephritis 1 year after the hospitalization reported here. Urine cultures became positive for both *E. coli* and a staphylococcus in patient 6. In no instance was a gram-negative organism isolated prior to the isolation of a staphylococcus. None of the patients was catheterized, nor were any genitourinary surgical procedures carried out prior to the time gram-negative bacilluria developed. Unfortunately, follow-up data are not available for the other two survivors.

A striking feature of this condition is the unique combination of infecting organism, the valve it attacks, and the type of narcotic involved. With the addition of this group, a total of 16 patients with bacterial endocarditis secondary to heroin addiction has now been reported. The causative organism has been a staphylococcus in 15 and *Pseudomonas* in 1. Of the staphylococcal cases, the tricuspid valve alone was involved in all five patients who have come to autopsy. Nine of the ten patients who survived are thought to have had tricuspid involvement because of a clinical picture identical to the autopsy-proved cases and the absence of any evidence of involvement of other valves.

This highly characteristic triad of heroin, the staphylococcus, and the tricuspid valve can be compared with the distinctly different situation in opium addicts. Luttgens (1949) reported 11 patients with endocarditis secondary to "main line" opium addiction, and briefly noted the salient features of two additional cases. A nonhemolytic penicillin-resistant streptococcus (authors' note: enterococcus?) was isolated in seven, *Streptococcus faecalis* in two, and *E. coli, Aerobacter aerogenes,* and *P. aeruginosa* from one patient each. The valvular involvement was primarily aortic in ten patients and mitral in two. In contrast to endocarditis associated with heroin addiction, only one patient had isolated tricuspid valvulitis. Luttgens also cited

five opium addicts with *Candida* endocarditis. This has not been reported in heroin addicts.

Of particular significance is the fact that septic pulmonary infarction is the dominant clinical feature of staphylococcal tricuspid endocarditis, and cardiac findings may be minimal or absent. In contrast to previously reported patients, systemic embolism was a constant finding. Five of the six patients had renal embolism. Hematogenous staphylococcal pyelonephritis may predispose to subsequent gram-negative pyelonephritis. This sequence was observed in three patients. Whereas heroin addicts characteristically have staphylococcal tricuspid endocarditis, opium and morphine addicts have predominantly left-sided endocarditis, and this is infrequently staphylococcal.

## Acknowledgment

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## Literature Cited

Bain, R. C., J. E. Edwards, C. H. Scheiffey, and J. E. Geraci. 1958. Right-sided bacterial endocarditis and endarteritis. A clinical and pathologic study. Am. J. Med. **24**:98.

De Navaques, S. 1956. Further studies in experimental pyelonephritis produced by various bacteria with special reference to renal scarring as a factor in pathogenesis. J. Pathol. Bacteriol. **71**:27.

Goldburgh, H. L., S. Baer, and M. M. Lieber. 1942. Acute bacterial endocarditis of the tricuspid valve. Am. J. Med. Sci. **204**:310.

Holmes, J. R., and M. J. Romansky. 1958. Successful short-term therapy of enterococcal and staphylococcal endocarditis with ristocetin: seven patients. Preliminary report. Antibiotics Ann. 1957-1958, p. 187.

Hussey, H. H., and S. Katz. 1945. Septic pulmonary infarction: report of 8 cases. Ann. Internal Med. **22**:526.

Hussey, H. H., and S. Katz. 1950. Infections resulting from narcotic addiction. Am. J. Med. **9**:186.

Hussey, H. H., T. F. Kelliher, B. F. Schaeffer, and B. J. Walsh. 1944. Septicemia and bacterial endocarditis resulting from heroin addiction. J. Am. Med. Assoc. **126**:126.

Luttgens, W. F. 1949. Endocarditis in "main line" opium addicts. Arch. Internal Med. **83**:653.

Wilder, R. M. Jr. 1957. Staphylococcal bacterial endocarditis: report of a case in a narcotic addict. Am. J. Med. **23**:325.

Wilhelm, F., H. L. Hirsh, H. H. Hussey, and H. F. Dowling. 1947. The treatment of bacterial endocarditis with penicillin. Ann. Internal Med. **26**:221.

# ANTIKIDNEY ANTIBODIES IN GLOMERULONEPHRITIS

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In the last 5 years, developments in the use of immunological techniques have progressed to the point that many techniques are available for the study of immunological phenomena. The relationship of immunological phenomena to the pathogenesis of glomerulonephritis has been investigated in both experimental animals and in patients, with varying results. This laboratory has previously reported studies utilizing a latex-particle agglutination method which produced few false positive reactions (Kramer et al., 1961). Preliminary studies indicated that although this technique was not valuable as an absolute diagnostic method, since only about 50% of patients with glomerulonephritis revealed elevated titers when tested, it may have value as a prognostic procedure.

## Materials and Methods

Sera from patients with suspect glomerulonephritis were examined by the antihuman kidney (AHK) antibody test, previously described (Kramer et al., 1961). Serial specimens were obtained at varying intervals throughout the course of the disease and examined for the presence of antibody. The diagnosis and progression of the glomerular pathology was determined, in a majority of cases, by serial kidney biopsy, where possible, and by clinical progression, as evidenced by examination of urinary sediment, 24 hr excretion of urine protein, antistreptolysin (ASO) titer, and measurement of renal function using inulin, para-aminohippuric acid (PAH), and urea-clearance techniques. Patients studied were from the George Washington University Medical Division, District of Columbia General Hospital, and the Medical Service, George Washington University Hospital.

## Results

During the study 37 patients were encountered in whom the diagnosis of glomerulonephritis was established beyond reasonable doubt (Table 1). Of this group, 28 patients manifested the presence of circulating AHK antibody and 9 did not. In this group of patients there are some in whom the diagnosis was chronic glomerulonephritis or subacute glomerulonephritis. In these, the clinical course

TABLE 1. *Summary of patients studied*

| Patients | Diagnosis* | AHK titer | | Outcome |
|---|---|---|---|---|
| | | Highest value | Duration | |
| 1 | CGN | 1:1280 | 2 wk | Death |
| 2 | SGN | 1:320 | 4 wk | Death |
| 3 | CGN | 1:1280 | 2 wk | Death |
| 4 | SGN | 1:160 | 3 months | Death |
| 5 | MGN | 1:640 | 5 months | Death |
| 6 | AGN | 1:40 | 11 months | Progression |
| 7 | AGN | 1:40 | 12 months | Progression |
| 8 | AGN | 1:320 | 2 wk | Death |
| 9 | AGN | 1:320 | 1 wk | Stable |
| 10 | AGN | 1:80 | 2 wk | Stable |
| 11 | MGM | 1:40 | 2 wk | Recovered |
| 12 | MGM | 1:40 | 4 wk | Recovered |
| 13 | AGN | 1:40 | 12 months | Healed and recurrance |
| 14 | AGN | 1:40 | 1 wk | Recovered |
| 15 | AGN | 1:40 | 1 wk | Recovered |
| 16 | AGN | 1:40 | 3 wk | Recovered |
| 17 | AGN | 1:40 | 2 wk | Recovered |
| 18 | AGN | 1:40 | 3 months | Recovered |
| 19 | AGN | 1:40 | 3 wk | Recovered |
| 20 | AGN | 1:20 | 2 wk | Recovered |
| 21 | MGM | 1:40 | 4 wk | Recovered |
| 22 | AGN | 1:40 | 4 wk | Recovering |
| 23 | AGN | 1:40 | 1 wk | Recovering |
| 24 | AGN | 1:20 | 4 wk | Recovering |
| 25 | AGN | 1:20 | 2 wk | Recovered |
| 26 | AGN | 1:20 | 2 wk | Recovered |
| 27 | AGN | 1:40 | 3 months | Recovered |
| 28 | AGN | 1:40 | 2 months | Recovered |
| 29 | AGN | Neg | – | Recovered |
| 30 | AGN | Neg | – | Recovered |
| 31 | CGN | Neg | – | Stable |
| 32 | AGN | Neg | – | Recovering |
| 33 | AGN | Neg | – | Recovered |
| 34 | AGN | Neg | – | Died, other causes |
| 35 | AGN | Neg | – | Recovered |
| 36 | AGN | Neg | – | Recovered |
| 37 | MGM | Neg | – | Recovered |

*Symbols used: AGN = acute glomerulonephritis; MGM = membranous glomerulonephritis; SGN = subacute glomerulonephritis; CGN = chronic glomerulonephritis; and Neg = negative.

appeared to be that of an acute glomerulonephritis, but the biopsy demonstrated pathological changes consistent with the diagnosis listed. Our criteria for the diagnosis of glomerulonephritis by biopsy have been presented in previous reports (Parrish, Watt, and Howe, 1957; Watt, Howe, and Parrish, 1959; Parrish et al., 1961).

In the 37 patients studied, we found that the 9 patients not showing presence of AHK antibody all demonstrated healing either clinically or on repeat biopsy. The remaining 28 patients could be subdivided into two groups: those showing AHK titers below 1:160 and those having titers of 1:160 and above (Table 2). Of the group exhibiting titers of 1:160 and above, six developed progressive glomerulonephritis and died within a year of the time they were first seen. In contrast, 19 of the 21 patients having titers below 1:160 showed healing or lack of progression of their disease in a follow-up period ranging from 6 months to 3 years. Thus, we feel that the height of the titer gives good indication as to whether the patient will develop progressive glomerulonephritis or not.

TABLE 2. *Relationship of height of titer to recovery*

| Patients | Titer | | | Total |
|---|---|---|---|---|
| | Negative | <1:160 | >1:160 | |
| Total | 9 | 21 | 7 | 37 |
| Recovered | 9 | 19 | 1 | 29 |
| Progressive glomerulonephritis | 0 | 2 | 6 | 8 |

The relation between duration of AHK antibody titer to recovery is not as clear-cut as that between height of titer and recovery (Table 3). In all, 30 patients maintained an elevation of titer for less than 70 days after onset of symptoms. In this group, 26 showed evidence of healing or stability of the lesions, and four demonstrated progression and eventual death. Those that died had an elevation of titer at the time of death and had a maximum rise in titer of 1:160 or higher. In the group of patients maintaining their titers for more than 70 days, three of seven recovered and four expired. These three patients had titers less than 1:160 and two had elevated titers for less than 90 days. The one patient, showing stability with a titer of 1:40 one year after his initial onset of symptoms, had a

TABLE 3. *Relationship of duration of titer to recovery*

| Patients | Time in days | | Total |
|---|---|---|---|
| | <70 | >70 | |
| Total | 30 | 7 | 37 |
| Recovered | 26 | 3 | 29 |
| Progressive glomerulonephritis | 4 | 4 | 8 |

second acute episode at the time of testing, following a group A streptococcal pyoderma. Figure 1 represents the composite picture of the relationship between height and duration of titer.

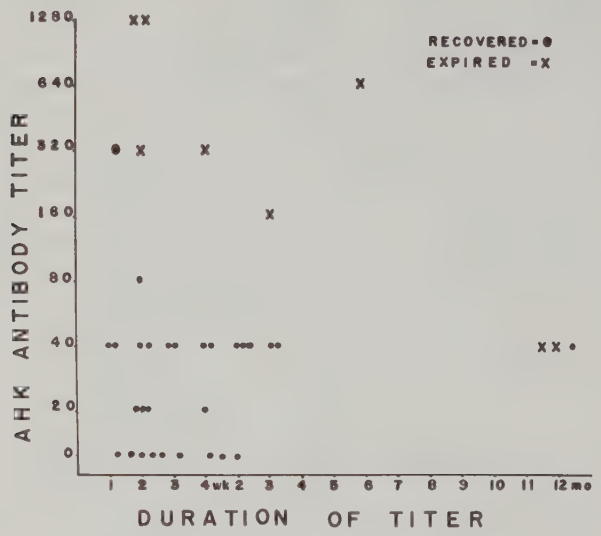


FIG. 1. *Relationship between course and AHK antibody titer.*

## Discussion

The role of an auto-antibody in the pathogenesis of glomerulonephritis was first suggested by Schwentker and Comploier (1939). Through the use of a mixture of homologous kidney and streptococcal toxins, they were able to demonstrate the presence of a complement-fixing antibody in immunized animals. However, they made no attempt to demonstrate similar antibodies in patients with glomerulonephritis. Lange et al. (1949), using a collodion particle agglutination, demonstrated the presence of a circulating antibody in patients with glomerulonephritis and felt that the presence of antibody may offer some insight into prognosis. With the development of hemagglutination techniques, Liu and McCrory (1958) also demonstrated a circulating antibody with a greater percentage of positive sera from patients with more progressive glomerulonephritis. Serum reactivity was noted in patients with lupus erythematosus and in those with acute infectious processes, but usually in a lower titer.

Further evidence for an immunological factor in glomerulonephritis has been obtained by Lange, Wasserman, and Slobody (1960) in their

studies of serum-complement changes in both patient material and in the experimental animal. They related a fall in serum complement to the development and progression of the glomerular lesion. In perfusion studies, Lange and Wenk (1954) demonstrated binding of complement in the kidneys of animals with experimental nephritis.

Experimental data of Krakower and Greenspoon (1951) showed that the antigen responsible for the AHK antibodies is located in the glomerular basement membrane, but it has not been further characterized. The immunological aspects of glomerulonephritis have been recently reviewed in detail with an excellent pro and con discussion of the role of an auto-immune process (Peters and Freedman, 1959).

Attempting to relate our data to the concept of an auto-immune pathogenesis for glomerulonephritis, we feel that the presence of circulating antibody demonstrated is an index of severity of the immunological assault. Whether it is the cause of the continuing progression of disease or not cannot be determined at present. There is a suggestion that, at some point in the developing glomerular lesion, self-perpetuation takes place and the disease becomes progressive and fatal. The continued presence of AHK antibody is a reflection of this progression and may represent the period of transition between the usual self-limiting poststreptococcal syndrome and the progressive form demonstrated here. The answer to this problem rests in further characterization of the antibody and of the antigen or antigens responsible for its production.

## Acknowledgments

This investigation has been carried out while under the tenure of a senior research fellowship (N.C.K.) and a postdoctoral research fellowship (E.E.B.) from the U.S. Public Health Service and has been supported by the following grants: H-3732 (C3), ASF-8509, and A-2234 (C3), from the National Institutes of Health, and WHA 23 from the Washington Heart Association.

## Literature Cited

Krakower, C. A., and S. A. Greenspoon. 1951. Localization of nephrotoxic antigen within the isolated glomeruli. A. M. A. Arch. Pathol. 51:629-632.

Kramer, N. C., M. F. Watt, J. S. Howe, and A. E. Parrish. 1961. Circulating antihuman kidney antibodies in human renal disease. Am. J. Med. 30:39-45.

Lange, K., M. M. A. Gold, D. Weiner, and V. Simon. 1949. Auto-antibodies in human glomerulonephritis. J. Clin. Invest. 28:50-55.

Lange, K., and S. Wenk. 1954. Investigations into the site of complement loss in experimental glomerulonephritis. Am. J. Med. Sci. 228:454-460.

Lange, K., E. Wasserman, and L. B. Slobody. 1960. The significance of serum complement levels for the diagnosis and prognosis of acute and subacute glomerulonephritis and lupus erythematosus disseminatus. Ann. Internal Med. 53:636-646.

Liu, C. T., and W. McCrory. 1958. Auto-antibodies in human glomerulonephritis and nephrotic syndrome. J. Immunol. **81**:492-498.

Parrish, A. E., M. F. Watt, and J. S. Howe. 1957. Membranous glomerulonephritis A. M. A. Arch. Internal Med. **100**:620-627.

Parrish, A. E., N. C. Kramer, F. E. Hatch. M. F. Watt, and J. S. Howe. 1961. The relation between glomerular function and histology in acute glomerulonephritis. J. Lab. Clin. Med. **58**:197-203.

Peters, J. H., and P. Freedman. 1959. Immunologic aspects of renal disease. N. Engl. J. Med. **261**:1166-1173, 1225-1235, 1275-1281.

Schwentker, F. F., and F. C. Comploier. 1939. Production of kidney antibodies by injection of homologous kidney plus bacterial toxins. J. Exptl. Med. **70**:223-230.

Watt, M. F., J. S. Howe, and A. E. Parrish. 1959. Tubular changes in acute glomerulonephritis. A. M. A. Arch. Internal Med. **103**:690-695.

# TREATMENT FACTORS OF BACTEREMIA CAUSED BY GRAM-NEGATIVE BACTERIA

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Infections caused by gram-negative bacilli have increased to the extent that they now present a serious hospital problem (Waisbren, 1951; Spittel, Martin, and Nichols, 1956; Weil and Spink, 1958; Finland, Jones, and Barnes, 1959). Although these bacteria have achieved less notoriety than the staphylococci, they are at the present time a significant hazard and a more frequent cause of hospital-acquired infections than *Staphylococcus aureus* at the University of Illinois Hospitals.

Bacteremia caused by gram-negative bacteria has a rapid and frequently fatal course. Diagnosis and therapeutic decisions usually must be made before precise bacteriological identification or antibiotic sensitivities are available. Proper management of this syndrome therefore requires a thorough understanding of factors influencing its course and development, and the relative merit of various therapeutic agents. To clarify these factors, all instances of "gram-negative" bacteremia observed at the University of Illinois Research and Educational Hospitals during an 8-year period were reviewed.

## Materials and Methods

The records of all patients diagnosed as having bacteremia caused by members of the families *Enterobacteriaceae* or *Pseudominadaceae* between 1 January 1951 and 31 December 1958 were examined. All premortem blood-culture isolates of these bacteria were reviewed and the hospital charts of these patients examined. Only those patients whose course was clinically compatible with gram-negative bacteremia were selected for further evaluation. These records were analyzed with reference to host factors predisposing to the development and outcome of bacteremia, evidences of specific bacterial virulence, and the effectiveness of various therapeutic agents. Particular attention was paid to the clinical response to antibiotic therapy and the in vitro antibiotic sensitivity of the etiological agent.

## Results

A total of 173 instances of gram-negative bacteremia, 73 of which were fatal, were recognized. A progressive increase in frequency was recognized during this period. By 1958, the annual occurrence rate had quadrupled, and gram-negative bacteremia was observed once per 263 hospital admissions. During the same period, mortality from gram-negative bacteremia doubled. This difference suggests that there has been increased recognition and more adequate therapy of this syndrome.

The frequency and fatality rates of bacteremia caused by individual species of gram-negative bacilli are illustrated in Fig. 1. Instances in which two species of gram-negative bacilli or a bacillus and a gram-positive or gram-negative coccus were isolated are termed mixed infections. The classification "other gram-negative bacilli" included *Salmonella typhimurium, S. cerro,* an unidentified bacillus, and one member each of the Providence and Alkalescens-Dispar groups.

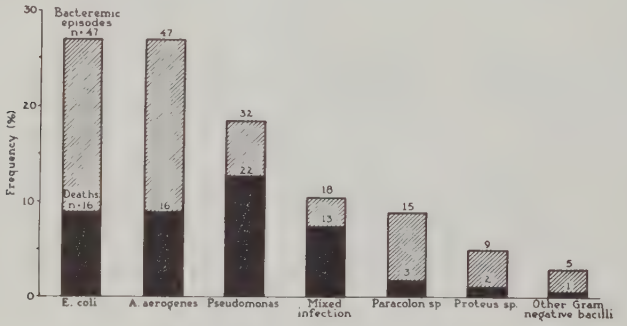


FIG. 1. *Frequency of specific gram-negative bacilli causing bacteremia. The relative occurence of bacteremia caused by individual species of gram-negative bacteria may be read on the vertical axis. The darker portions of the bars depict the fatality rate of each bacterial species.*

Certain bacterial species appear to be more lethal than others. Although the fatality associated with *Pseudomonas* (72%) and mixed species (69%) was much greater than that caused by the other species of gram-negative bacilli (31%), no specific differences in bacterial virulence could be demonstrated when host factors were considered.

Table 1 demonstrates the paramount importance of host status as a determinant of the outcome of gram-negative bacteremia, and that this factor obscured any differences in bacterial virulence.

TABLE 1. *Fatality in gram-negative bacteremia and relation to etiologic agent and host status*

| Bacterial species | Underlying host disease | | |
|---|---|---|---|
| | Rapidly fatal | Ultimately fatal | None or Nonfatal |
| *Escherichia coli* | 7/8* | 9/12 | 0/27 |
| *Aerobacter aerogenes* | 3/4 | 9/21 | 4/22 |
| *Pseudomonas* sp. | 10/10 | 11/14 | 1/8 |
| Paracolon sp. | - | 1/3 | 2/12 |
| *Proteus* sp. | - | 2/2 | 0/7 |
| Other gram-negative bacilli | - | 1/1 | 0/4 |
| Mixed species | 1/1 | 10/12 | 2/5 |
| Total | 21/23 (91%) | 43/65 (66%) | 9/85 (11%) |

*Fatalities/number of patients with bacteremia.

To evaluate resistance and other host factors, patients were placed into three categories of underlying host disease, depending on the severity of their primary illness. Patients who had either acute leukemia or blastic relapse of chronic leukemia were classified as having "rapidly fatal disease." Only 2 of the 23 patients in this category recovered from bacteremia. The "ultimately fatal disease" group was composed of 65 patients whose underlying disease was of such severity that it would probably be fatal within the next 4 years. The fatality rate of bacteremia in these patients was 66%. Patients classified as having "nonfatal disease" had an underlying illness which was not apt to prove fatal within 4 years; of this group, only 11% succumbed to the episode of bacteremia. Since a variety of underlying diseases was observed, patients were placed into the latter two groups solely on the severity of their disease rather than by specific diagnosis.

This material indicates that the apparently greater virulence of Pseudomonas and mixed infections actually resulted from their predilection for producing bacteremia in more severely ill patients. A significantly greater number of *Pseudomonas* and mixed infections occurred in patients with rapidly fatal or ultimately fatal underlying disease than did infections caused by the other species of gram-negative bacilli ($X^2 = 14$; $P < 0.001$). It is conceivable that if a larger sample size were observed *Pseudomonas* and mixed infections might prove to be significantly more lethal, but this could not be verified from the present data.

Sixty-five (38%) of the patients were receiving antibiotics at the time they developed bacteremia, and 23 (13%) had completed a course of antibiotic treatment within the 2 weeks immediately preceding the bacteremia. Antibiotic therapy prior to the onset of bacteremia was observed most often in the more seriously ill patients; 78% of patients with rapidly fatal underlying disease and 56% of those with ultimately fatal disease were on antibiotic prophylaxis or had recently received antibiotic therapy, but only 39% of those with nonfatal

underlying disease were receiving or had recently received antibiotics when they developed bacteremia. Within the subgroups of underlying host disease, antibiotic prophylaxis had no detectable effect on mortality rates. It was quite clear, however, that prophylaxis did not prevent gram-negative bacteremia. No conclusions could be made regarding the effect of antibiotics in reducing or increasing the frequency of gram-negative bacteremia. Prior or concomitant antibiotic therapy did favor the occurrence of *Pseudomonas*, *Paracolon*, and *Aerobacter aerogenes* as etiologic agents.

Forty-one patients were receiving an average of 200 mg of cortisone or its equivalent as therapy for their primary disease at the onset of gram-negative bacteremia. An additional 20 patients received adrenal steroids as an adjunct to the treatment of bacteremia; 112 received no steroid therapy. These patients were carefully studied for clarification of the value of steroid therapy in gram-negative bacteremia. Steroids were not discontinued during the course of bacteremia in any patients who were receiving them at the onset of bacteremia. Higher fatality rates occurred in each category of underlying host disease among patients pretreated with steroids than in those who did not receive steroids prior to the development of bacteremia (Table 2). This difference was not significant among patients with rapidly fatal or ultimately fatal underlying disease, because of the extreme lethality of bacteremia in these patients. The difference was significant [$X^2$(Yates modification) = 3.95; $P < .05$], however, among patients with nonfatal underlying disease.

TABLE 2. *Effect of steroids on fatality from gram-negative bacteremia*

| Steroid treatment | Number of patients | Fatalities | |
|---|---|---|---|
| | | | % |
| *Patients with rapidly fatal disease* | | | |
| Steroid treatment before bacteremia | 18 | 94 | |
| No steroid treatment before bacteremia | 5 | 80 | |
| Steroid treatment after bacteremia | 1 | 100 | |
| No steroid treatment after bacteremia | 4 | 75 | |
| *Patients with ultimately fatal disease* | | | |
| Steroid treatment before bacteremia | 16 | 75 | |
| No steroid treatment before bacteremia | 49 | 63 | |
| Steroid treatment after bacteremia | 12 | 83 | |
| No steroid treatment after bacteremia | 37 | 57 | |
| *Patients with nonfatal disease* | | | |
| Steroid treatment before bacteremia | 8 | 37 | |
| No steroid treatment before bacteremia | 77 | 8 | |
| Steroid treatment after bacteremia | 7 | 43 | |
| No steroid treatment after bacteremia | 70 | 4 | |

The pattern observed in patients who received adrenal steroids as an adjunct to therapy of bacteremia was quite similar. In each

group of patients the mortality of those treated with steroids was greater than that observed in patients who did not receive steroids; among patients with nonfatal underlying disease, this difference was statistically significant [$X^2$(Yates modification) = 8.13; $P < .01$].

Since it has been postulated that the mechanism of endotoxin lethality in experimental animals is at least partially due to shock, the effect of adrenal steroids administered before the onset of bacteremia in preventing shock was investigated. Table 3 illustrates the incidence of shock among patients who received steroids before the development of bacteremia and in patients who were not receiving steroids. Only 28% of patients who were not receiving steroids at the time of bacteremia developed shock, but 63% of those who were receiving steroid therapy developed shock ($X^2 = 14$; $P < .001$). The frequency of shock was similar in patients treated with steroids before the onset of bacteremia, irregardless of the category of underlying host disease into which they had been classified. Similarly, the severity of the primary illness did not appear to be related to the development of shock in patients not pretreated with steroids, since the incidence of shock was not significantly different among these patients in each of the three categories of underlying host disease.

TABLE 3. *Frequency of shock* from gram-negative bacteremia in relation to steroid administration*

| Steroid treatment | Number of patients | Patients with shock |
|---|---|---|
| | | % |
| *Patients with rapidly fatal disease* | | |
| Steroid treatment before bacteremia | 17 | 65 |
| No steroid treatment before bacteremia | 5 | 20 |
| *Patients with ultimately fatal disease* | | |
| Steroid treatment before bacteremia | 16 | 62 |
| No steroid treatment before bacteremia | 48 | 33 |
| *Patients with nonfatal disease* | | |
| Steroid treatment before bacteremia | 7 | 57 |
| No steroid treatment before bacteremia | 75 | 25 |

*BP- 90/60 or a drop of 60 mm mercury in patients with documented hypertension.

The role of antibiotic therapy in the outcome of gram-negative bacteremia is demonstrated in Fig. 2. Analysis of individual antibiotics is not presented because the variety and combinations of antibiotics used make interpretation difficult. Instead, the results of antibiotic therapy were evaluated solely on the basis of in vitro antibiotic sensitivity of the infecting organism to the antibiotic or antibiotics administered. Eight patients received antibiotic therapy for less than 24 hr before death or died of causes unrelated to persistent infection and are omitted from these tabulations.

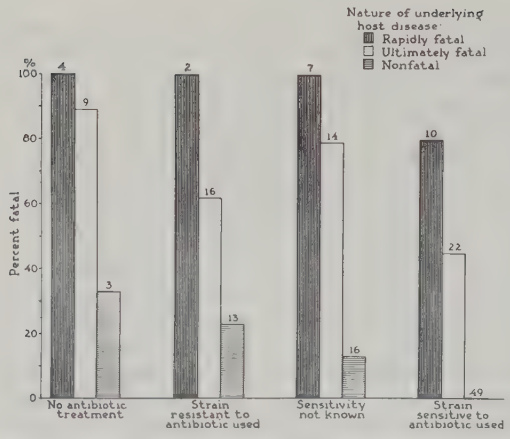


FIG. 2. *Relation of bacterial antibiotic sensitivity and host status to fatalities caused by gram-negative bacteremia. The antibiotic sensitivity of the infecting bacteria to the antibiotic administered is illustrated on the abscissa. Fatality rates in each category of underlying host disease shown by the vertical bars.*

Sixteen patients received no antibiotic therapy, 81 patients were treated with an antibiotic to which the infecting organism was sensitive, and 31 received an antibiotic to which the infecting organism was resistant by in vitro tests. The antibiotic sensitivity of the infecting microorganism was not determined in 37 instances, and it was not possible to determine the appropriateness of antibiotic therapy in these patients.

The nature of the underlying host disease appeared to be as important a determinant as antibiotic therapy. There were but two survivors of the 23 patients with rapidly fatal disease and bacteremia, and both of these were patients who received an antibiotic to which the infecting bacteria were sensitive.

Only one of the nine patients with ultimately fatal disease who did not receive antibiotic therapy survived. In contrast, 12 of the 22 patients who received appropriate antibiotic therapy survived. Only 38% of those patients who received an antibiotic judged ineffective in vitro, and 21% of those in whom the antibiotic sensitivity of the infecting organism was not known, survived.

Fatalities were considerably less in patients with nonfatal underlying disease. No fatalities occurred among the 49 patients who received appropriate antibiotic therapy, but one of the three patients who received no antibiotics died. The mortality of patients treated with an antibiotic of undetermined effectiveness was 11%; it was 23% in those patients receiving an antibiotic to which the infecting organism was resistant.

Tetracycline and chloramphenicol appeared to be the most effective individual antibiotics. Combinations of antibiotics effective in vitro did not exhibit a greater therapeutic effect than a single effective antibiotic. The use of a combination of antibiotics did not result in the selection of an antibiotic effective in vitro any more frequently than did the use of a single antibiotic.

## Discussion

A number of investigations (Waisbren, 1951; Spittel et al., 1956; Weil and Spink, 1958; Finland et al., 1959) have emphasized the increasing frequency of gram-negative bacteremia and have clarified some of the problems associated with these infections. This study was undertaken to amplify these observations and to elucidate factors which might significantly influence the outcome of gram-negative bacteremia.

The paramount importance of host status as a determinant of the outcome of bacteremia was the most striking feature of this investigation. Host factors obscured any differences in specific bacterial virulence; although some species appeared to be more lethal, this cannot be confirmed without additional observations.

Antibiotic prophylaxis had no demonstrable effect on fatality rates, but did select certain bacterial species as etiologic agents. Prophylactic treatment may predispose to the development of gram-negative bacteremia, but studies of antibiotic prophylaxis in a large homogenous hospital population are necessary for verification of this problem.

The use of adrenal steroids in the management of gram-negative bacteremia has been vigorously championed on the basis of both clinical impressions and experimental observations (Weil and Spink, 1958; Spink, 1960; Spink and Anderson, 1954; Smith and Thomas, 1954). This clinical impression has not been confirmed by controlled studies, and studies of pneumonia treated with ACTH clearly document the hazard of such assumptions (Kass, Ingbar, and Finland, 1950). Patients in the latter study had persistent bacteremia and extension of pulmonary infection despite defervescence and clinical improvement following ACTH administration.

Prior administration of steroids prevents the lethal effects of endotoxin in experimental animals (Spink and Anderson, 1954; Smith and Thomas, 1954), but this was not demonstrated in humans. Patients who had been previously treated with steroids had a significantly greater fatality rate and were more prone to develop shock than untreated patients. It is unlikely that this predisposition to shock could be due to iatrogenic adrenal insufficiency since these patients were receiving an average of 200 mg of cortisone or its equivalent daily. Treatment with steroids after the onset of bacteremia appeared to have altered mortality unfavorably. Although it

is possible that more severe bacteremia prompted steroid treatment, this possibility could not be verified or disproven from the records.

The discrepancy between the therapeutic benefit of steroids administered to animals prior to endotoxin challenge and the deleterious effect in humans may be due to differences in the two types of challenge. Experimental endotoxin shock involves a single challenge with dead bacterial products. Bacteremic shock may well be caused by endotoxins, but in addition to endotoxemia, there is also bacteremia with live, virulent bacilli capable of continuing proliferation. Experimental viral and bacterial infections in animals are uniformly potentiated by concomitant steroid administration (Kass and Finland, 1957). The use of steroids in human infections has been justified by the reversal of this deleterious effect by effective antibiotic therapy, but these experiments differ considerably from gram-negative bacteremia where an antibiotic proven to be effective in vitro was administered in only 50% of the episodes.

Antibiotics were an important factor in recovery, but were no more important than host factors. The importance of host factors in determining the outcome of treatment of staphylococcal bacteremia has been emphasized (Lepper et al., *in press*). The present data strongly support this contention and indicate that any valid consideration of antibacterial therapy must include evaluation of host factors.

The results of antibiotic therapy correlated closely with in vitro sensitivity determinations. In each group of underlying host diseases, an antibiotic shown to be effective in vitro provided maximal therapeutic benefit. An effective antibiotic afforded the only hope of survival from bacteremia in patients with rapidly fatal underlying disease and completely protected patients with nonfatal underlying disease. Administration of an ineffective antibiotic did not preclude recovery from bacteremia, nor did an effective antibiotic insure recovery. A few patients recovered without antibiotic therapy.

Antibiotics were usually administered as an emergency procedure before bacteriological identification or antibiotic sensitivity testing was completed. Knowledge of previous antibiotic therapy, portals of entry, and prior cultures of localized infections were invaluable guides for the selection of an appropriate antibiotic.

## Literature Cited

Finland, M., N. F. Jones, and M. N. Barnes. 1959. Changes in the occurrence of serious bacterial infections. Trans. Assoc. Am. Phys. **72**:305-321.

Kass, E. H., and M. Finland. 1957. Adrenocortical hormones and the management of infection. Ann. Rev. Med. **8**:1-18.

Kass, E. H., S. H. Ingbar, and M. Finland. 1950. Effects of adrenocorticotropic hormone in pneumonia, clinical, bacteriological and serological studies. Ann. Internal Med. **33**:1081-1098.

Smith, R. T., and L. Thomas. 1954. Effect of cortisone on response to endotoxin in mature rabbits. Proc. Soc. Exptl. Biol. Med. **86**:810-813.

Spink, W. W. 1960. The pathogenesis and management of shock due to infection. Arch. Internal Med. **106**.433-442.

Spink, W. W., and D. Anderson. 1954. Experimental studies on the significance of endotoxin in the pathogenesis of brucellosis. J. Clin. Invest. **33**:540-548.

Spittel, J. A., Jr., W. J. Martin, and D. R. Nichols. 1956. Bacteremia owing to gram-negative bacilli: experiences in the treatment of 137 patients in a 15-year period. Ann. Internal Med. **44**:302-315.

Waisbren, B. A. 1951. Bacteremia due to gram negative bacilli other than Salmonella. A. M. A. Arch. Internal Med. **88**:467-488.

Weil, M. H., and W. W. Spink. 1958. The shock syndrome associated with bacteremia due to gram-negative bacilli. A. M. A. Arch. Internal Med. **101**:184-193.

# DEGRADATION OF ENDOTOXIN BY SPLENIC EXTRACTS

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Detoxification of bacterial endotoxin by the reticuloendothelial system has been demonstrated by showing that, 5 min after injecting endotoxin into the dog's spleen, all the effluent blood and a homogenized suspension of spleen were nontoxic by the chick-embryo test at the end of this period (Smith and Thomas, 1956). Detoxification did not occur if the dog had been exposed to hemorrhagic shock for 2 hr or had received a sublethal dose of Thorotrast prior to injection of the endotoxin (Wiznitzer et al., 1960).

This preliminary report presents data from in vitro experiments designed to identify the process of detoxification.

## Materials and Methods

A $P_{32}$-labeled endotoxin of *Escherichia coli* 0111:B4 was added to saline extracts of fresh spleen. Samples of the resulting mixtures were assayed for toxicity by the chick-embryo test. Protein fractions of other samples were localized by electrophoresis, and bound radioactivity was determined by scanning the electrophoregrams.

Spleen, liver, kidney, and skeletal muscle from dogs killed by exsanguination were homogenized in 0.85% sterile saline (4 C) with a Virtis tissue disintegrator, to a concentration of 0.5 g/ml. The homogenized suspensions were centrifuged at 4 C for 20 min at 2,000 rev/min; 1 ml of the clear supernatant of each suspension or 1 ml of serum was incubated at 37 C with 0.5 mg of labeled endotoxin. Samples of these mixtures were tested for toxicity at 0 time, and after intervals of 5 min, 30 min, and 1 hr. (In some of these toxicity experiments we used unlabeled endotoxin. Sensitivity of the method was 1 $\mu$g in 0.1 ml. Occasional endotoxin preparations were positive with much less than 1 $\mu$g. Toxicity was considered present

if five of seven chick embryos inoculated with the same sample were killed. Toxicity was considered absent if no more than two of seven embryos were killed.) Each experiment was done three to ten times. Other samples removed after identical periods of incubation were placed in *duplicate* in a Spinco hanging strip electrophoresis cell. Electrophoresis was carried out on Whatman 3MM paper for 16 to 17 hr in Veronal buffer (pH 8.6, ionic strength 0.075), using a constant current of 0.25 ma per cm width of paper. At the completion of electrophoresis, the two paper strips were processed as follows: One was stained with bromophenol blue to localize the protein fractions, which were quantitated with a Spinco Analytrol densitometer; the second was scanned for localization and quantitation of radioactivity, using a chromatogram scanner equipped with a flat slide drive system.

Control experiments performed with each tissue extract included samples of toxin alone, toxin with tissue extract at zero time, and toxin with spleen extract maintained at 4 C for 1 hr.

Similar experiments were carried out on the same tissues from dogs subjected to irreversible hemorrhagic shock.

## Results

*Toxicity.* Endotoxin alone incubated for 1 hr at 37 C, or endotoxin immediately after being mixed with extracts of spleen, liver, kidney, skeletal muscle, or serum, or endotoxin mixed with these extracts and held at 4 C for 1 hr was uniformly toxic, i.e., it was lethal for at least five of seven chick embryos.

Endotoxin incubated at 37 C with spleen extract from healthy dogs resulted in detoxification within 5 min (Table 1). If reacted for 1 hr the percentage of embryos killed was the same (Table 2).

TABLE 1. *Detoxification of endotoxin by tissue extracts of normal dogs (incubation for 5 min at 37 C)*

| Expt. no. | Toxin | Toxin + spleen | Toxin + liver |
|---|---|---|---|
| 1 | 6* | 2 | 6 |
| 2 | 7 | 2 | 6 |
| 3 | 7 | 3 | 5 |

*The figures indicate the number of chick embryos killed out of a total of seven tested with the same sample.

This was not the case with liver extracts or serum. Incubation with liver for 5 min was not effective; but if incubation was continued for

1 hr there was partial detoxification. Kidney and skeletal muscle extracts after 1 hr of incubation failed to destroy endotoxin. Extracts of spleen, liver, and serum from shocked dogs did not detoxify endotoxin (Table 3). This was true whether the shock was reversible or irreversible to transfusion.

TABLE 2. *Detoxification of endotoxin by tissue extracts from normal dogs (incubation for 1 hr at 37 C)*

| Expt. no. | Toxin | Toxin + spleen | Toxin + liver | Toxin + kidney or muscle | Toxin + serum |
|---|---|---|---|---|---|
| 1 | 6* | 2 | | | |
| 2 | 7 | 3 | | | |
| 3 | 7 | 2 | 3 | 6 | 6 |
| 4 | 6 | 3 | 5 | 5 | 6 |
| 5 | 6 | 2 | 2 | 5 | 5 |
| 6 | 7 | 2 | 3 | 4 | |
| 7 | 6 | 1 | 4 | 6 | 5 |
| 8 | 6 | 1 | 4 | 6 | 6 |
| 9 | 7 | 2 | 3 | 5 | 5 |

*The numbers indicate the number of chick embryos killed out of a total of seven tested with the same sample.

TABLE 3. *Detoxification of endotoxin by tissue extracts of dogs after 6 hr of hemorrhagic shock (incubation for 1 hr at 37 C)*

| Expt. no. | Toxin | Toxin + spleen | Toxin + liver | Toxin + serum |
|---|---|---|---|---|
| 1 | 7* | 5 | 5 | |
| 2 | 7 | 7 | 7 | |
| 3 | 7 | 7 | | |
| 4 | 6 | 6 | 7 | 7 |

*The figures indicate the number of chick embryos killed out of a total of seven tested with the same sample.

*Release of* $P_{32}$. The electrophoregram of the $P_{32}$-labeled endotoxin alone showed activity only in the vicinity of the origin, indicating little or no migration of the toxin under the conditions employed (Fig. 1). The peak at the origin was accompanied by a second smaller peak of radioactivity immediately cathodal to the origin. Incubation with a normal splenic suspension reduced the total radioactivity ($P_{32}$ release) and eliminated the smaller peak. Radioactivity was also observed in the area of the $\alpha$ and $\beta$ globulins. Incubation with a normal liver suspension reduced the radioactivity at the origin by

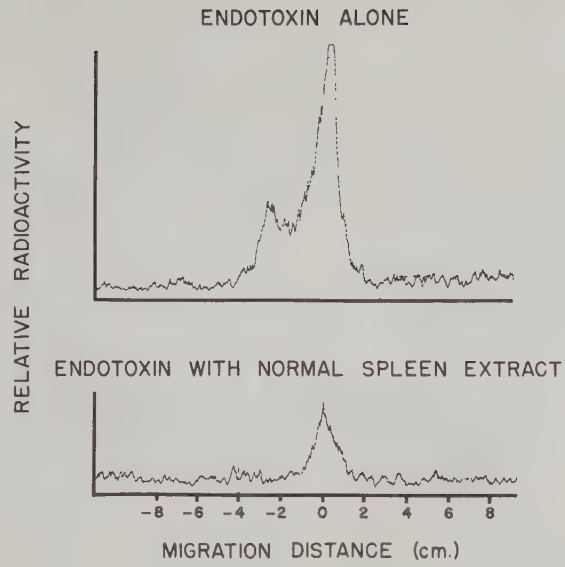


FIG. 1. *Electrophoresis of endotoxin alone (top) and endotoxin plus normal spleen extract (bottom).*

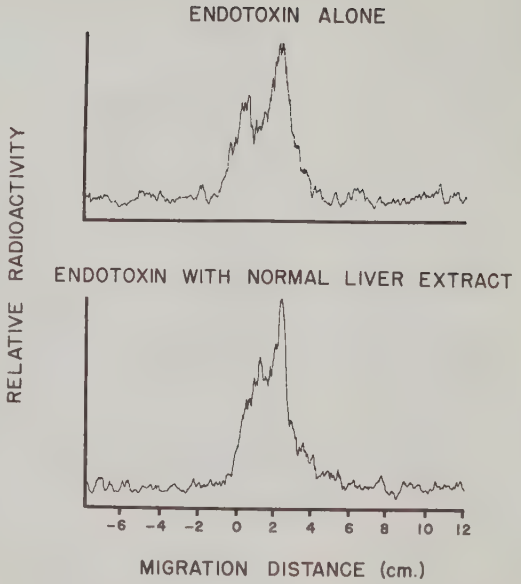


FIG. 2. *Electrophoresis of endotoxin alone (top) and endotoxin plus normal liver extract (bottom).*

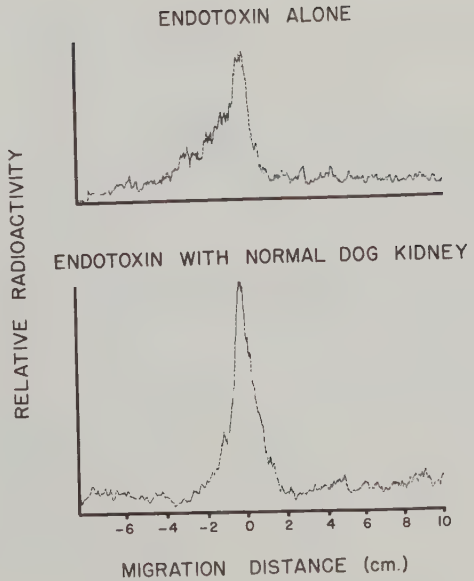


FIG. 3. *Electrophoresis of endotoxin alone (top) and endotoxin plus kidney extract.*

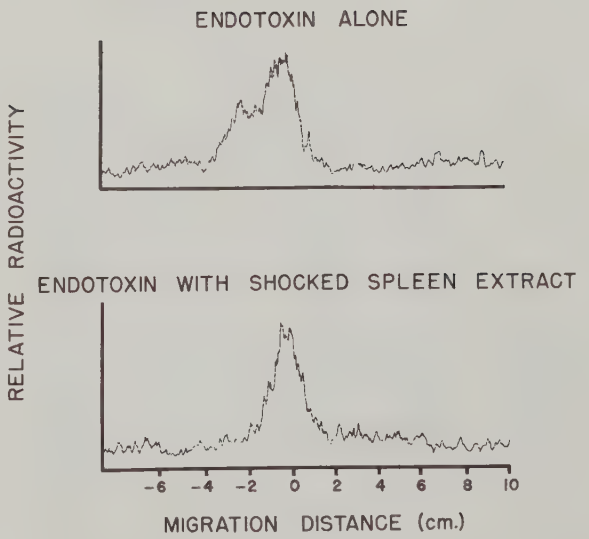


FIG. 4. *Electrophoresis of endotoxin alone (top) and endotoxin plus spleen extract from a "shocked" dog.*

30% of that achieved by the splenic suspension (Fig. 2). Incubation of endotoxin with serum for the same period produced still less effect. No significant effect was produced by kidney (Fig. 3), skeletal muscle extract, or shock spleen suspension (Fig. 4).

*Protein fractions.* A comparison of protein fractions showed a decrease in albumin in serum of shocked animals and an increase in $\alpha$ and $\beta$ globulins in serum, spleen, and liver extracts.

## Discussion

Various patterns of degradation of endotoxin with differing experimental models have been reported. Westphal *(personal communication)* demonstrated that detoxification by alkaline hydrolysis eliminates the secondary peak in the ultracentrifugation pattern. Tal and Goebel (1950) observed that detoxification by mild acid hydrolysis coincides with a 50% decrease in phosphorus content. Enzymatic degradation has been observed by Rowley (1956), who noted detoxification in serum and coincident release of $P_{32}$ after incubation for 1 hr at 37 C and pH 8.4. Skarnes (1961), using the Ouchterlony agar diffusion method and immunoelectrophoretic techniques, observed the loss of a diffusion band with detoxification after a 1 hr incubation of a similar preparation. Ho and Kass (1958) reported that prolonged incubation with normal human, rat, or rabbit serum reduces the lethality in the rat and chick embryo. But the kinetics of these enzymatic reactions are not likely to be closely related to those in vivo enzymatic reactions which are significant for defense in acute infections. On the other hand, the ability of aqueous extracts of spleen to detoxify endotoxin almost immediately signifies that such a system is more likely to be the one involved in such defense. Further experiments are in progress to identify and isolate the detoxifying enzymes and the products of degradation of endotoxin.

## Literature Cited

Ho, M., and E. H. Kass. 1958. Protective effect of components of normal blood against the lethal action of endotoxin. J. Lab. Clin. Med. **51**:297-311.

Rowley, D. 1956. Some factors affecting the resistance of animals to bacterial infection. Ann. N. Y. Acad. Sci. **66**:304-311.

Skarnes, R. C. 1961. Studies on endotoxin altered by plasma or alkaline hydrolysis. Bacteriol. Proc., p. 131.

Smith, R. T., and L. Thomas. 1956. Lethal effect of endotoxins on the chick embyro. J. Exptl. Med. **104**:217-231.

Tal, C., and W. Goebel. 1950. On the nature of the toxic component of the antigen of Shigella paradysenteriae type 2 (Flexner). J. Exptl. Med. **92**:25-31.

Wiznitzer, T., N. Better, W. Rachlin, N. Atkins, E. D. Frank, and J. Fine. 1960. In vivo detoxification of endotoxin by the reticulo-endothelial system. J. Exptl. Med. **112**:1157-1166.

# EFFECT OF MITHRAMYCIN AND OTHER ANTIBIOTICS ON A HUMAN EPIDERMOID CARCINOMA GROWING IN NEWBORN SWISS MICE

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Previous experimentation (Merker, Anido, and Bowie, 1960) revealed that a human epidermoid carcinoma, H.Ep. #3, which is capable of growing progressively for 12 to 14 days in newborn Swiss mice, was not remarkably affected by chemotherapeutic efforts. Recently, however, tests revealed that mithramycin, a relatively new antibiotic, had the ability to inhibit the human tumor as it grew in newborn Swiss mice. The antitumor properties of this antibiotic were first described by Rao, Cullen, and Sobin (1960). In view of their encouraging experimental data, clinical toxicity and chemotherapy studies have been undertaken (Curreri, 1960; Curreri and Ansfield, 1960; Parker, Wiltsie, and Jackson, 1960).

The present paper deals with the effects of mithramycin and other antibiotics on the growth of H.Ep. #3 in neonatal mice.

## Materials and Methods

Tumor suspensions for inoculations were prepared from 7- to 10-day-old H.Ep. #3 tissue grown intramuscularly in X-irradiated and cortisone-treated rats (Toolan, 1954). Tumors were removed from surrounding muscle, minced with scalpels, and then mixed with an equal volume of physiological saline fortified with penicillin (1,000 units/ml) and streptomycin sulfate (2 mg/ml). The tissue was gently homogenized by hand, and 0.1 ml of the dispersion was injected subcutaneously through a 23-gauge needle.

Recipients were Swiss mice less than 24 hr old. Newborn were obtained from randomly bred Swiss mice kept in breeding pens containing five females and one male. Several days prior to term, females were removed to individual cages. After birth, mother and newborn were housed in the same cage throughout the course of the

experimentation. An average litter consisted of 9 to 10; therefore an experimental group could consist of 4 to 5 treated (marked by cutting the tip off the tail) and 4 to 5 untreated.

Newborns, inoculated with tumor within 24 hr after birth, were not handled again until they were 7 days old. At that time they were weighed, and tumors measured by vernier calipers. Two measurements were made, at right angles to each other; the two values were then added and an average diameter (in cm) determined. The mother was fed, *ad libitum*, a standard laboratory chow supplemented with sunflower seeds. Wood shavings were used as bedding.

Antiobiotics were generally prepared in 0.86% sodium chloride. When required, as with actinomycin D and mitomycin C, stock solutions in absolute ethyl alcohol for the former and 0.86% sodium chloride for the latter were prepared and kept at 5 C. Working solutions were prepared by diluting the stock solution with saline. Final concentrations of all antibiotic solutions used for therapy were adjusted so that a single dose was contained in a volume of approximately 0.1 ml.

Therapy consisted, in all cases, of single, daily, intraperitoneal injections starting on the 7th day after birth and inoculation with tumor. Treatment was then continued for four additional days for a total of five injections.

## Results

Detailed data on growth and regression of tumors and the effect that tumor implants can have on newborn mice will be published elsewhere. Only data pertaining to the chemotherapy system will be discussed.

Table 1 presents average mortality figures. In general, surviving tumor-bearing newborn mice appeared healthy, and actively growing tumor implants had little significant adverse effect on host body-weight gain during the period critical to the chemotherapy studies. Surviving mice allow a rather high percentage of tumors to "take."

TABLE 1. *Mortality of neonatal mice inoculated (subcutaneously) with H. Ep. #3*

| Days after transplantation | Tumor "takes" | Avg tumor diameter* | Avg body wt* | Mortality |
|---|---|---|---|---|
| | % | *cm* | *g* | % |
| 7† | 89 (260 of 291) | 0.56-0.94 | 3.8-6.1 | 15 (43 of 279) |
| 12 to 14‡ | 93 (139 of 149) | 0.82-1.37 | 5.5-10.3 | 3 (4 of 119) |

*Groups of 3 to 6 newborn.

†Data prior to experimentation on 20 to 30 litters.

‡Saline-injected control.

When tumors failed to increase in size between the 7th day (initial measurement) and the 12th to 14th days, they were classified "static," and when tumors decreased in size they were designated "regressing." After the 14th day, tumor growth was usually less vigorous, and, because of the heterologous nature of the tumor-host relationship, tissue eventually regressed.

Because of eventual tumor regression, the chemotherapy test was limited to a "short-term" period of observation which extended from the 7th day, at which time treatment was started, to the 14th day, when treatment ended. Additional observations were made up to the 19th day, but this was done primarily for the purpose of determining any delayed mortality and toxicity caused by drug treatments. If, as occasionally happened, tumors did increase in size after the 14th day, such information was noted and added to the final evaluation.

For the purpose of establishing limits of drug effectiveness, average tumor diameters derived from groups of three to six newborns were plotted on a logarithmic scale. A central value (grand average) and upper and lower limits were then drawn in by inspection. Figure 1 presents data from 45 consecutive bioassays to illustrate the range of variation obtained with this tumor system. Based on these data, a 0.6-cm average tumor diameter for a group of three to six treated tumors would lie outside the 95% confidence limit, and, in relation to the grand average (central value) of 1.05 cm, this 0.6 would represent an inhibition of 40 to 45%. Therefore, antibiotics and other chemicals, which were to be judged as effective antitumor agents in this baby-mouse system, were expected to inhibit tumor growth at least to that extent.

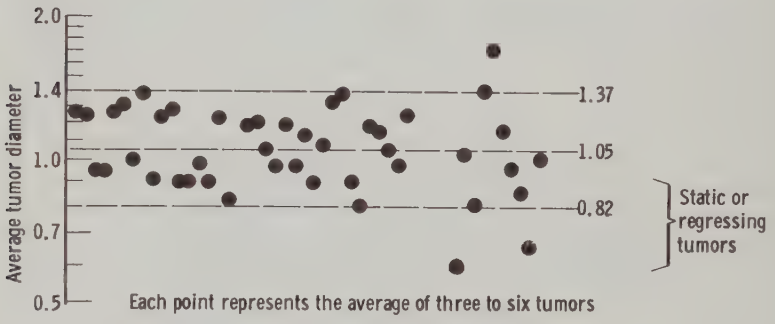


FIG. 1. *Growth of H. Ep. #3 in neonatal mice (12 to 14 days).*

A detailed set of data on azaserine is presented in Table 2 to illustrate the type of information obtained for each antibiotic tested.

Observations were made on the number of dead or missing animals, body weight, number of tumors, average tumor diameter, regressing tumors, and static tumors. At this dose of azaserine (15 mg/kg daily for 5 days), lethal effects developed progressively from the 12th day through the 19th day of observation, and body weight gain was also adversely affected. It is obvious, therefore, that azaserine at 15 mg/kg is lethal to newborn mice bearing human tumors. In spite of this toxicity, there was no marked inhibitory effect against tumor growth, nor did the drug affect the number of regressing and static tumors.

TABLE 2. *Effect of azaserine on the growth of H. Ep. #3 in neonatal mice**

| Days post implant | Dead or missing | | Average body weight and range | | Number of tumors | | Average tumor diameter and range | | Regressed tumors | | Static tumors | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | C | T | C | T | C | T | C | T | C | T | C | T |
| 7 | 0/3 | 0/4 | 5.2 (5.1-5.4) | 4.6 (4.4-5.2) | 3/3 | 4/4 | 0.76 (0.6-0.9) | 0.78 (0.75-0.85) | | | | |
| 9 | 0/3 | 0/4 | 6.8 (6.6-7.0) | 5.8 (4.8-6.5) | 3/3 | 4/4 | 0.95 (0.7-1.1) | 0.97 (0.85-1.05) | 0/3 | 0/4 | 0/3 | 0/4 |
| 12 | 0/3 | 1/4 | 9.1 (9.0-9.3) | 6.1 (4.5-7.6) | 3/3 | 3/3 | 1.20 (0.95-1.35) | 1.21 (1.1-1.3) | 0/3 | 0/3 | 0/3 | 0/3 |
| 14 | 0/3 | 2/4 | 10.3 (9.8-10.8) | 8.1 | 3/3 | 1/1 | 1.25 (0.85-1.45) | 1.50 | 1/3 | 0/1 | 0/3 | 0/1 |
| 19 | 0/3 | 3/4 | 10.7 (9.8-11.4) | Dead | 2/3 | Dead | 1.32 (1.30-1.35) | Dead | 2/2 | Dead | | |

*Dose: 15 mg/kg daily, given intraperitoneally starting on the 7th day. C: saline injected tumor bearers (control). T: drug treated. Number of tumors: number of tumor-inoculated newborn having measurable tumors. Regressed tumors: tumors which decreased in size from the previous measurement. Static tumors: tumors which failed to increase in size from previous measurement.

It is in this manner that all other antibiotics were tested. Doses were regulated upwards or downwards by factors of 2 or $\frac{1}{2}$ until suitable assay results could be obtained. Two or more trials were performed at all dose levels.

Table 3 presents data on eight antibiotics of past or current interest to clinical and experimental groups. Of the eight antibiotics,

TABLE 3. *Response of H. Ep. #3 in various test systems to antibiotic therapy*

| Antibiotic | Newborn | | Cortisone-treated mice* | | In vitro-in vivo† | |
|---|---|---|---|---|---|---|
| | Effect | Dose | Effect | Dose | Effect | Concn |
| | | *mg/kg* | | *mg/kg* | | *mg/ml* |
| Actidione | – | 2.5 | – | 30 | – | 0.3 |
| Miracil D | – | 25 | ±/T | 62.5 | – | 0.87 |
| Azaserine | – | 15 | – | 10 | – | 0.14 |
| Actinomycin D | – | 0.1 | ±/T | 0.06 | + | 0.002 |
| Mitomycin C | – | 2.0 | ±/T | 2.5 | + | 0.02 |
| Actinobolin $SO_4$ | – | 250 | – | 1500 | – | 14.0 |
| 8-Azaguanine | – | 75 | ±/T | 60 | – | 1.05 |
| Mithramycin | ± | 0.25 | ±/T | 0.5 | + | 0.0035 |

*The mice were 21 to 30 days old and weighed 18 to 20 g.

†Host animal for tumor implant after in vitro incubation; cortisone-treated Swiss mice.

seven were classified as ineffective, while one, mithramycin, was designated as moderately effective at the specified dose of 0.25 mg/kg. The degree of effectiveness of mithramycin listed in Table 3 is given as $\pm$, or moderate. This partial inhibition is shown in Fig. 2. Inspection of the figure reveals that on the 12th to 14th days, tumors in treated baby mice were inhibited by approximately 40 to 45%, a value previously designated as being within the limits expected for an active compound. Since tumors were not completely inhibited, they tended in this experiment to increase in size beyond the 14th day. Host body weight data revealed no significant weight losses. However, histological examination of organs obtained from other groups of tumor-bearing baby mice, treated with this dose of mithramycin, revealed that the antibiotic causes damage to the liver.

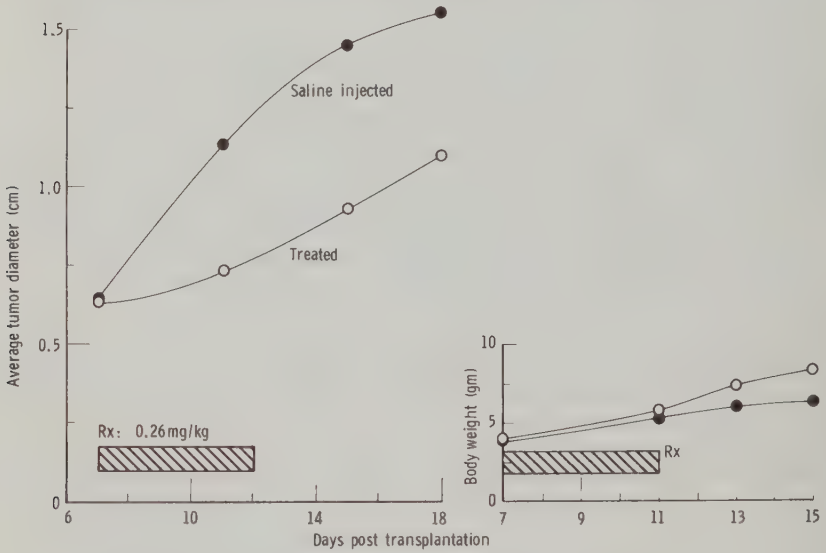


FIG. 2. *Effect of mithramycin on H. Ep. #3 growing in newborn mice.*

At higher doses of mithramycin, i.e., 0.5 mg/kg, a correspondingly greater antitumor effect was observed. Data presented in Table 4 illustrate the marked inhibitory effect and the regressions obtained. Data on host body weight in the four experiments illustrate that differences were observed. However, these may only have been a reflection of the differences in amount of tumor tissue present at that time rather than a reflection of drug toxicity. Although the drug at this dose may not have adversely affected host carcass weight, mortalities were encountered in experiments 1 and 4.

TABLE 4. *Effect of mithramycin on the growth of H. Ep. #3 in neonatal mice**

| Experiment number | Mortality | | Avg body weight | | Avg tumor diameter | | Regressing tumors | |
|---|---|---|---|---|---|---|---|---|
| | T | C | T | C | T | C | T | C |
| | | | *g* | *g* | *cm* | *cm* | | |
| 1 | 2/4 | 0/3 | 5.4 | 7.9 | 0.82 | 1.27 | 2/2 | 0/3 |
| 2 | 0/3 | 0/3 | 6.6 | 8.4 | 0.23 | 1.33 | 3/3 | 0/3 |
| 3 | 0/4 | 0/4 | 5.7 | 6.8 | 0.52 | 1.19 | 3/4 | 0/4 |
| 4 | 1/3 | 0/2 | 5.9 | 8.0 | 0.0 | 1.32 | 3/3 | 0/2 |

*Treatment dose: 0.5mg/kg daily for 5 days. Final observations made on the 12th to 14th days after mice were born. H. Ep. #3 tissue inoculated when animals were less than 24 hr old. Treatment started on the 7th day. T: drug treated. C: control.

The anti-H.Ep. #3 activity of mithramycin was confirmed by in vivo tests (Table 3) which used cortisone-treated mice in a test procedure previously described (Merker et al., 1960*b*). Other compounds which have inhibited H.Ep. #3 when the cortisone-treated mouse was used as host for the tumor have been Miracil D, actinomycin D, mitomycin C, and 8-azaguanine. However, these antibiotics as well as mithramycin have always produced their effects in association with some degree of host toxicity.

In the in vitro-in vivo tests, a direct action of mithramycin against the human tumor tissue could be demonstrated by incubating a sample of minced tumor with a solution of the drug prior to inoculating the tissue into host animals. A full description of the method will be published elsewhere; suffice it to state at this time that a concentration of 0.0035 mg/ml of mithramycin resulted in the complete inhibition of tumor growth (Table 3). In this respect, only mitomycin C and actinomycin D have displayed such activity, with actinomycin D having approximately the same degree of potency.

In view of the heterologous nature of the H.Ep. #3-newborn mouse system, it was of interest to test mithramycin against other types of tumors growing in newborn mice to determine if the newborn host represented a peculiar metabolic background against which transplanted tumors would always respond to therapy. Two transplantable animal tumors, which were known not to respond to mithramycin therapy when grown in their natural hosts, were used in these studies: the Jensen rat sarcoma and mouse sarcoma S 180. The Jensen rat sarcoma was found to be quite adaptable for growth in the newborn mouse. Several assays were attempted and one of them is presented in Fig. 3. It can be seen that mithramycin was not inhibitory to tumor growth. The mouse sarcoma, S 180, was less amenable for drug studies because the transplanted tissue was very lethal to neonatal animals. In general, over 90% of inoculated mice

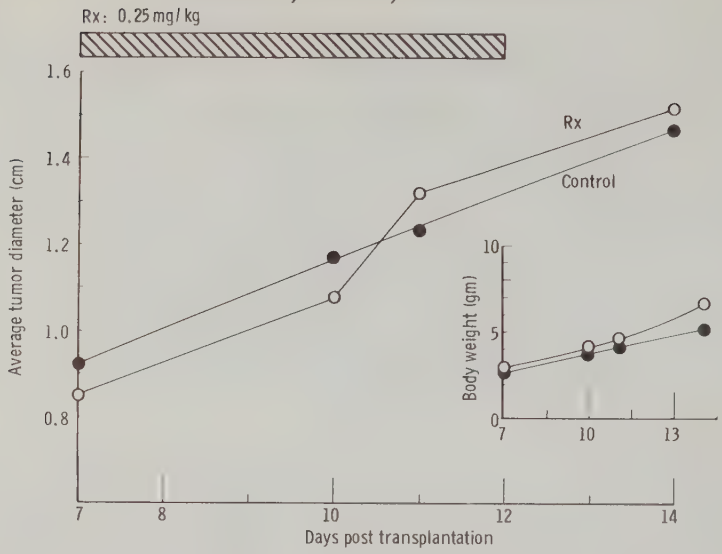


FIG. 3. *Effect of mithramycin on Jensen rat sarcoma growing in newborn mice.*

were dead within a 14-day period. Occasionally, sufficient mice survived to allow for a proper observation to be made on the effect of the antibiotic on tumor growth. One such assay is presented in Fig. 4 to illustrate the lack of effect of mithramycin on the growth of the mouse sarcoma. Therapy had no remarkable effect on survival time of tumor-bearing mice.

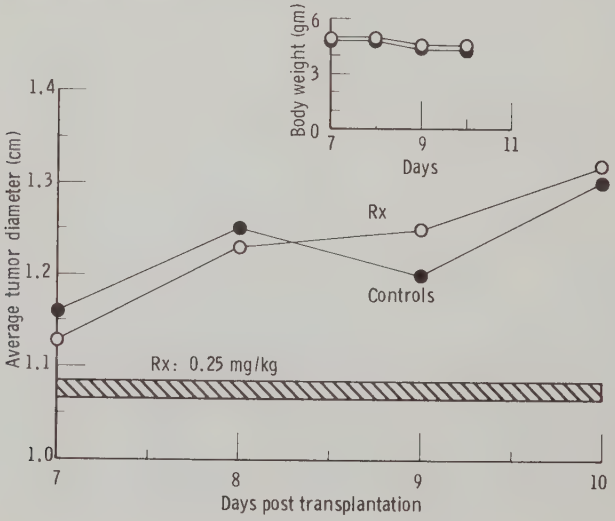


FIG. 4. *Effect of mithramycin on S 180 growing in newborn mice.*

TABLE 5. *Biological activity of mithramycin in newborn and weanling animals bearing growing tumors*

| Tumor | Host animal | |
|---|---|---|
| | Suckling mice* | Weaned mice |
| H. Ep. #3 | + | +† |
| Jensen rat sarcoma | – | –† |
| Sarcoma 180 | – | – |

*Weight: 4 to 10 g, 7 to 12 days old.
†Weight: 18 to 20 g, 21 to 30 days old, cortisone-treated.

A comparison of the biological responsiveness of the three tumors, as they grew in various conditioned and nonconditioned animal hosts, is made in Table 5. For purposes of conciseness, complete test data are not presented, and a qualitative, rather than a quantitative, rating has been used to designate biological activity. This comparison illustrates the point that the human epidermoid carcinoma was responsive to mithramycin therapy when the tumor was grown in newborn mice and cortisone-treated adult mice, whereas the two animal tumors, not of the same histological type as the human tumor, were nonresponsive when grown in newborn mice.

## Discussion

Antibiotic substances usually have been tested for anticancer activity against transplantable animal tumors growing in adult animals (Sugiura et al., 1958). Recently, systems using human tumors growing in X-irradiated or cortisone-treated laboratory animals have also been used to test antibiotic substances for anticancer activity (Merker et al., 1960*b*; Teller and Palm, 1957; Teller et al., 1958). Since the only nonconditioned host environment used routinely to grow human tumors and to conduct chemotherapy trials has been the chicken egg, it was thought advisable to investigate other animal hosts. Investigations of Patti and Moore (1950), Gheorghiu (1926), and Bullock (1915) suggested the possibility of using the less-than-24-hr-old animal as a recipient for human tumor transplants. Preliminary investigations (Merker et al., 1960*a*) revealed the feasibility of using the neonatal mouse, not only for tumor growth studies but also for drug-testing purposes. Chemotherapy data revealed that the chemicals and antibiotics tested were not very effective against the human epidermoid carcinoma growing in the newborn. However, test data presented in this paper reveal that a relatively new antibiotic, mithramycin, does have the capacity to inhibit tumor growth.

The fact that mithramycin has anti-H.Ep. #3 activity confirms the observations of Rao et al. (1960). They observed that this antibiotic was effective against the growth of a transplantable human sarcoma (H.S. #1) growing in conditioned rats, and a transplantable mouse tumor, adenocarcinoma 755. Although this can imply that the antibiotic may have universal activity against all transplantable tumors, data presented in this paper indicate that mithramycin did not inhibit the growth of two well-known transplantable animal tumors, the Jensen rat sarcoma and the mouse sarcoma, S 180.

The nonresponsiveness of the two transplantable animal tumors was confirmed in tests conducted with newborn mice. The fact that the antibiotic was ineffective against the Jensen rat sarcoma, when the newborn mouse was used as host, reveals that the anti-H.Ep. #3 activity of mithramycin was not simply due to the heterologous relationship of the human tumor and the newborn host.

Microscopic examination of selected organs of baby mice treated with mithramycin revealed that no particular pathology was observed in the spleen, small intestines, or stomach. However, liver sections displayed marked focal necrosis, extensive parenchymatous degeneration, diffuse interstitial round cell infiltration (predominantly composed of monocytes and lymphocytes), and very extensive periportal inflammatory cell infiltration (composed of monocytes, lymphocytes, polynuclear leukocytes, and macrophages). These observations agree with the findings of Olson *(personal communication)*, who observed liver damage in dogs treated with mithramycin, and with the clinical findings reported by Curreri and Ansfield (1960), which revealed that the antibiotic did have a marked damaging effect on the liver.

In view of the toxic effects produced against the newborn host, one cannot completely rule out the possibility that the anti-H.Ep. #3 activity of mithramycin is in some way related to the toxic influences on the host. However, in vitro-in vivo test data revealed potent antitumor activity, indicating that mithramycin does have the capability of acting directly against the tumor cell. This observation is in agreement with the findings of Rao et al. (1960), who observed that the antibiotic inhibited the growth of HeLa cells in tissue culture.

Other antibiotics tested against the H.Ep. #3-newborn mouse system have not been active tumor inhibitors even though they may have displayed some degree of activity against the tumor in tests conducted in the cortisone-treated mouse or in the in vitro-in vivo tests. The lack of correlation between test data obtained from the newborn mouse and other systems may be due to the fact that therapy in the newborn-mouse system starts after tumors are well established, whereas treatment is started 24 hr after transplantation when the cortisone-treated host is used. Other considerations that may account for the lack of correlation may be the differences in detoxification (Michael, Giesel, and Sutherland, 1960) and excretion mechanisms (McCance, 1948) which have been found between newborns or

young and older animals. The fact that the host can indeed be involved in the response is indicated by the fact that H.Ep. #3, when grown in the conditioned rat, does not respond to mithramycin therapy (Olson, *personal communication).*

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## Literature Cited

Bullock, W. F. 1915. Heterologous transplantation. Mouse tumors in rats. Lancet 1:701-703.

Curreri, A. R. 1960. PA-144 toxicity studies. Proc. Am. Assoc. Cancer Research 3:103.

Curreri, A. R., and F. J. Ansfield. 1960. Mithramycin - human toxicology and preliminary therapeutic investigation. Cancer Chemotherapy Repts. No. 8, p. 18-22.

Gheorghiu, J. 1926. Heterologous cancer grafts; the growth of mouse cancer in rats. J. Pathol. Bacteriol. 29:171-176.

McCance, R. A. 1948. Renal function in early life. Physiol. Revs. 28:331-348.

Merker, P. C., P. Anido, and M. Bowie. 1960*a*. The effect of chemical agents on human epidermoid carcinoma (H. Ep. #3) growing in nonconditioned newborn Swiss mice. Proc. Am. Assoc. Cancer Research 3:133.

Merker, P. C., F. K. Pearce, J. S. Sarino, and G. W. Woolley. 1960*b*. The effect of streptonigrin and other antibiotics on a human epidermoid carcinoma, H. Ep. #3, growing in conditioned Swiss mice. Antibiotics & Chemotherapy 11:184-189.

Michael, A. F., R. G. Giesel, and J. M. Sutherland. 1960. Chloramphenicol toxicity in newborn rats. Antibiotics & Chemotherapy 10:368-370.

Parker, G. W., D. S. Wiltsie, and C. B. Jackson. 1960. The clinical evaluation of PA-144 (mithramycin) in solid tumors of adults. Cancer Chemotherapy Repts. No. 8, p. 23-26.

Patti, J., and A. E. Moore. 1950. Heterologous growth of sarcoma 180 with progression to death of hosts. Cancer Research 10:674-678.

Rao, K. U., W. P. Cullen, and B. A. Sobin. 1960. Mithramycin: an antibiotic with anti-tumor properties. Proc. Am. Assoc. Cancer Research 3:143.

Sugiura, K., C. C. Stock, H. C. Reilly, and M. M. Schmid. 1958. Studies in a tumor spectrum. VII. The effect of antibiotics on the growth of a variety of mouse, rat and hamster tumors. Cancer Research 18:66-77.

Teller, M. N., and J. E. Palm. 1957. Antitumor effects of several antibiotics on transplantable human tumors in the conditioned rat. Proc. Am. Assoc. Cancer Research 2:255.

Teller, M. N., P. C. Merker, J. E. Palm, and G. W. Woolley. The effect of actinobolin on the growth of human tumors H.S. #1 and H. Ep. #3 in the rat. Antibiotics Ann. 1958-1959, p. 518-521.

Toolan, H. W. 1954. Transplantable human neoplasm maintained in cortisone-treated laboratory animals: H.S. #1; H. Ep. #1; H. Ep. #2; H. Ep. #3; and H. Emb. Rh. #1. Cancer Research 14:660-666.

# ANTIBIOTIC SENSITIVITY OF EHRLICH ASCITES CELLS GROWN IN TISSUE CULTURE

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The inhibitory effects of a number of antibiotic substances on the growth of established cell lines in tissue culture have been reported from a number of laboratories (Eagle and Foley, 1956, 1958; Toplin, 1959; Smith, Lummis, and Brady, 1959; Perlman et al., 1959). Among the cell lines used in these studies were Earle's L cells (NCTC929), Eagle's KB line, and Gey's HeLa line. Eagle and Foley (1958) showed that these and other cell lines, derived from normal tissue and tumorous tissue, did not differ in their sensitivity to a number of cytotoxic agents, including antibiotics. In selecting cell lines for use in screening for cytotoxic agents, potentially useful in inhibiting growth of tumors, many have chosen for their experimental systems cell lines which grow well in tissue culture media. These may have lost their tumor-forming properties as determined by implantation of these cells into animals. We have studied the response to antibiotics of a cell line derived from Ehrlich ascites carcinoma cells taken from mice. This cell line, which has retained virulence during growth in tissue culture (over a 2-year period), is approximately as sensitive to antibiotics as Earle's L cells (NCTC 929).

## Materials and Methods

The Ehrlich ascites and Earle's L cells were grown in suspension culture in Waymouth's medium MB752/1 supplemented with 10% (v/v) calf serum and 0.03% (w/v) Methocel as described in our earlier publications (Perlman et al., 1959; Jackson, Giuffre, and Perlman, 1960; Giuffre, Perlman, and Jackson, 1961). In these experiments, 15 ml of inoculated media (containing 100,000 cells per ml) and dilutions of the antibiotics were added to 3/4 × 6 inch screw-capped test tubes. Samples were removed from the tubes and cell density determined using a Coulter Electronic Counter. The tubes were inserted in a Rollordrum apparatus (New Brunswick Scientific Company, Model TC-5), rotating at 55 to 60 rev/min and located in

a 37 C incubator. Cell densities were determined after 3 days incubation, and the cell multiplication factors (i.e., final count/initial count) were computed. Dose response curves were constructed, and the 50% inhibitory doses ($ID_{50}$) were determined graphically.

## Results and Discussion

The $ID_{50}$ values of a number of antibiotics, as determined in experiments with the Ehrlich ascites cell lines and the Earle's L cells, are summarized in Table 1. These two cell lines show similar responses to most of the antibiotics as far as could be determined in this experimental system.

TABLE 1. *Antibiotic inhibition of cell multiplication*

| Antibiotic | Approximate concentration causing 50% inhibition of multiplication | |
|---|---|---|
| | *Ehrlich ascites cells* | *Earle's L cells (NCTC929)* |
| | *mg/ml* | *mg/ml* |
| Antimycin A | 6 | 1 |
| Actinomycin B (mixture) | 4 | 5 |
| Actinomycin F | 8 | 10 |
| Streptovitacin A | 6 | 15 |
| Nonactin | 25 | 25 |
| Cinerubin B | 30 | 30 |
| Cycloheximide | 25 | 33 |
| Tubercidin | 40 | 40 |
| Porfiromycin | 100 | 300 |
| Puromycin | 1,200 | 4,000 |

The virulence of this Ehrlich ascites cell line was tested in Swiss mice several times during the course of the antibiotic-sensitivity tissue culture study. Tumors were found within 7 days in 40% of the mice injected with 1,000 cells per mouse. These tumor cells multiplied when passed from mouse to mouse. In studies of the virulence of "mouse-grown" cells, tumors formed within 4 days in 75% of mice injected with 1,000 cells per mouse. These results on the virulence of strains of Ehrlich ascites cells are in general agreement with those reported by Cailleau and Costa (1961), who found that injection of tissue culture-grown cells resulted in tumor formation in significant numbers when 1,000 cells were implanted per mouse.

In designing a tissue culture screening method for selection of antitumor substances, it is desirable to use a cell line which readily forms tumors when the cells, grown in vitro, are implanted into

animal hosts. Although these Ehrlich ascites cells are less virulent than cells maintained by passage in mice, they are many times more virulent than the other cell lines we have maintained in tissue culture. It seems possible that compounds with high cytotoxicity towards tumor-producing cells and low cytotoxicity towards "normal" cells may be selected by using a screen based on the response of this Ehrlich ascites cell line and Earle's L cells. The data in Table 1 show a threefold difference in sensitivity of the two cell lines to the less toxic substances tested, although no significant differences were found in response to the other antibiotics.

## Acknowledgments

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## Literature Cited

Cailleau, R., and F. Costa. 1961. Long-term *in vitro* cultivation of some mouse ascites tumors: Ehrlich ascites carcinoma. J. Natl. Cancer Inst. **26**:271-281.

Eagle, H., and G. E. Foley. 1956. Cytotoxic action of carcinolytic agents in tissue culture. Am. J. Med. **21**:734-749.

Eagle, H., and G. E. Foley. 1958. The cytotoxicity of anti-tumor agents for normal and animal cells in first tissue culture passage. Cancer Research **18**:1012-1016.

Giuffre, N. A., D. Perlman, and P. W. Jackson. 1961. Effect of medium composition on sensitivity in suspension culture of Earle's L-929 mouse fibroblasts to antibiotics. Cancer Chemotherapy Repts., no. 11, p. 57-60.

Jackson, P. W., N. Giuffre, and D. Perlman, 1960. Growth of Ehrlich ascites tumor cells in tissue culture. Can. J. Biochem. Physiol. **38**:1377-1378.

Perlman, D., N. A. Giuffre, P. W. Jackson, and F. E. Giardinello. 1959. Effects of antibiotics on multiplication of L cells in suspension culture. Proc. Soc. Exptl. Biol. Med. **102**:290-292.

Smith, C. G., W. L. Lummis, and J. E. Grady. 1959. An improved tissue culture assay. II. Cytotoxicity studies with antibiotics, chemicals, and solvents. Cancer Research **19**:847-852.

Toplin, I. 1959. Preliminary report on a tissue cytotoxicity test for large-scale cancer screening. Cancer Chemotherapy Repts., no. 3, p. 12-15.

# ANTITUMOR ACTION OF THE QUINOXALINE ANTIBIOTICS

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Since levomycin (Carter, Schaffner, and Gottlieb, 1954) and actinoleukin (Ueda et al., 1954) were reported in 1954, several similar polypeptide antibiotics have been described. From the results of degradation experiments, these antibiotics contain the same quinoxaline moiety. Therefore, the generic name "quinoxaline antibiotic" has been proposed for these agents (Kuroya et al., 1961).

During the course of antitumor screening of antibiotics from *Streptomyces* by one of us (Katagiri, at the Shionogi Research Laboratory, Osaka, Japan), about 2% of the culture filtrates produced quinoxaline antibiotics. From these culture filtrates, quinomycins A, B, and C, and triostin C were isolated and crystallized (Yoshida and Katagiri, 1961; Shoji and Katagiri, 1961).

The physicochemical and biological properties of these antibiotics are compared and summarized in Tables 1 and 2 (Shoji and Katagiri, 1961; Yoshida and Katagiri, 1961). Few differences in the physicochemical characteristics of these antibiotics have been observed.

TABLE 1. *Physicochemical properties of quinoxaline antibiotics*

| Antibiotic | Melting point (decomp) | Analysis | | | | | $[\alpha]_D$ | Ultraviolet spectrum $E\left(\frac{1\%}{1\ cm}\right)$ | |
|---|---|---|---|---|---|---|---|---|---|
| | | C | H | N | O | S | | | |
| | *C* | % | % | % | % | % | | | |
| Quinomycin A (echinomycin) | 221-223 | 53.98 | 6.39 | 14.51 | | 5.30 | -295.0 | 242.5 (622) | 322.5 (100) |
| Quinomycin B | 220.5-221.5 | 54.65 | 6.26 | 14.16 | | 6.05 | -300.0 | 242.5 (678) | 322.5 (117) |
| Quinomycin C | 218-220 | 55.50 | 6.50 | 13.78 | 16.70 | 5.30 | -250.6 | 242.5 (633) | 322.5 (114) |
| Triostins | 209-213 | 54.63 | 6.29 | 13.85 | | 5.70 | -144.5 | 242.5 (726) | 322.5 (117) |
| Triostin C | 210-214 | 55.40 | 6.42 | 13.86 | 16.35 | 5.53 | -133.4 | 242.5 (622) | 322.5 (115) |

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On the other hand, biological characteristics, especially the $LD_{50}$ in mice (inbred Swiss albino, dd strain) showed differences.

TABLE 2. *Biological properties of quinoxaline antibiotics*

| Organism | Minimal inhibitory concn (μg/ml) | | | |
|---|---|---|---|---|
| | Quinomycin A | Quinomycin B | Quinomycin C | Triostin C |
| *Staphylococcus aureus* | 0.10 | 0.02 | 0.02 | 0.10 |
| *Bacillus subtilis* | 0.02 | 0.01 | 0.01 | 0.05 |
| *Sarcina lutea* | 0.02 | 0.005 | 0.005 | 0.05 |
| *Escherichia coli* | >100 | >100 | >100 | >100 |
| *Shigella dysenteriae* | 1.0 | 2.0 | 2.0 | >100 |
| *Clostridium feseri* | 0.01 | 0.001 | 0.001 | 0.001 |
| *HeLa cells* | 0.01 | 0.001 | 0.001 | 0.01 |
| $LD_{50}$* | 0.4 | 0.04 | 0.04 | 100 |

*Intraperitoneally in mice (mg/kg).

The structure of echinomycin (quinomycin A was identified with echinomycin, a sample of which was kindly provided by Dr. Corbaz of Zurich) was determined (Fig. 1) by Keller-Schierlein, Mihailvic, and Prelog (1959). From structural studies of the quinoxaline antibiotics, the following differences were suggested (Otsuka and Shoji, *in press*). First, the basic difference between the triostins and the quinomycins is that the dithian ring of the latter is replaced by N,N-dimethylcystine in the triostins. Second, the differences between quinomycins A, B, and C and triostins A, B, and C are at the N-methyl amino acid site. Thus, quinomycin A and triostin A have N-methyl valine, and quinomycin B and triostin B have N-methyl isoleucine. This structure in quinomycin C and triostin C has not yet been firmly established, but indications are that it is dimethyl leucine.

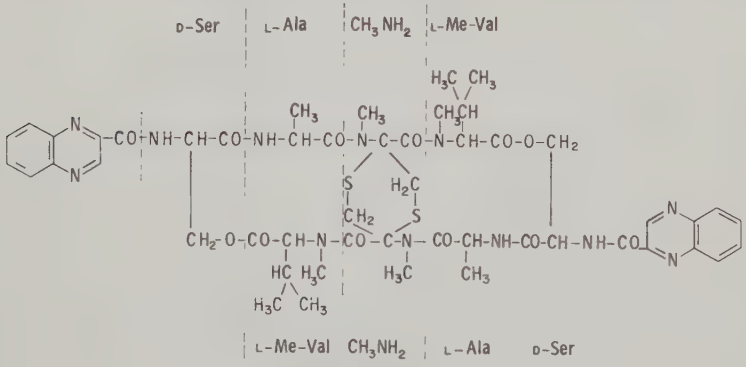


FIG. 1. *Structure of quinomycin A (echinomycin).*

Among these quinoxaline antibiotics, actinoleukin and antibiotic 6270 were reported to have antitumor activity (Preobrazhenskaya et al., 1958).

This report consists of observations on the effect of quinomycins and triostins on the growth of 31 solid tumors, 2 ascites tumors, and 1 virus leukemia.

## Materials and Methods

Because of a limited supply of triostin C, we used a mixture of triostins (40% A, 60% C, with a trace of B) in the present study.

Quinomycins were first dissolved in small volumes of ethanol and further diluted in sterile distilled water. Triostins were suspended in 0.5% carboxymethylcellulose in aqueous 0.85% NaCl.

The methods employed in the chemotherapy studies were as follows: for the solid tumors (Sugiura and Stock, 1952), subcutaneous implantations of tumors (small pieces of tumor, 2 $mm^3$) into healthy young animals (18 to 22 g mice, 80 to 100 g rats and hamsters) were carried out by the usual trocar method with a single implant into the right axillary region. In every set of experiments, tumor-bearing animals were divided into two groups, one of controls and the other for treatment with antibiotics. The progress of the tumors in the animals was recorded graphically by measuring two diameters with calipers at weekly intervals for 4 weeks after tumor transplantation.

In the case of ascites tumors (Sugiura, 1953), the intraperitoneal injection of 0.1 ml of the fluid containing about 1 million cancer cells was made into the inguinal region of each mouse by means of a $\frac{1}{4}$-ml syringe fitted with a 27-gauge needle. These mice regularly develop large amounts of milky ascites in 7 to 14 days and die in 10 to 20 days.

With the Friend virus leukemia (Sugiura, 1959), the intraperitoneal injection of 0.2 ml of a 10% homogenized suspension of leukemic spleens in saline was made into the inguinal region of each mouse. The effects of the compounds were evaluated by comparison of the weights of spleens in treated and untreated infected mice 3 weeks after injection of the leukemic inoculum. At this time, the spleens of leukemic mice weigh about 2.0 g; spleens of normal female Swiss albino mice (about 8 weeks old) weigh about 0.18 g.

All animals were maintained on a standard pellet diet (Purina Laboratory Chow) and water *ad libitum*.

Intraperitoneal injection of the antibiotics at or near maximal tolerated doses was begun 24 hr after inoculation with tumor material and was continued once daily for 7 days.

Test groups consisted of five animals; the data presented are averages of multiple experiments. The controls for these experiments uniformly showed 100% "takes" and rates of spontaneous regression of about 5%.

The degree of inhibition of growth of the solid tumors was graded according to the following scheme: -, no effect (tumor growth to $\frac{3}{4}$ or more of the diameter of the controls); $\pm$, slight inhibition (tumor growth from $\frac{1}{2}$ to $\frac{3}{4}$ the diameter of the controls); +, moderate inhibition (tumor growth from $\frac{1}{4}$ to $\frac{1}{2}$ the diameter of the controls); ++, marked inhibition (growth of $\frac{1}{4}$ or less of the average diameter of controls); +++, complete inhibition or destruction of tumors.

The degree of inhibition of ascites tumor growth was graded according to the following scheme: -, no effect (the fluid volume increased to $\frac{3}{4}$ or more the controls, 10 to 25 ml ascites); $\pm$, slight inhibition (the fluid volume increased to about $\frac{1}{2}$ the volume of the controls, 5 to 9 ml ascites); +, moderate inhibition (the fluid volume increased to about $\frac{1}{4}$ the volume of the controls, 1 to 4 ml ascites); ++, marked inhibition (no abdominal distention, no gross ascites); +++, complete inhibition or destruction of ascites.

The degree of inhibition of virus leukemia was graded according to the following scheme: -, no effect (no change in leukemic spleen weight); $\pm$, slight inhibition (about 25% spleen weight inhibition); +, moderate inhibition (about 50% spleen weight inhibition); ++, marked inhibition (about 75% spleen weight inhibition); +++, complete inhibition (no alteration of spleen weight from normal nonleukemic animals).

## Results

The results obtained with quinomycins A, B, and C and triostins are shown in Table 3. All results were evaluated at the end of the second week except in the cases of Friend virus leukemia, Iglesias ovarian tumor, and Iglesias adrenal tumor, which were evaluated at the 3rd, 5th, and 9th week, respectively.

It is clear that the repeated daily injections of 0.01 mg/kg of quinomycin C had a destructive effect on Ehrlich ascites carcinoma, a marked inhibitory effect on sarcoma 180 ascites tumor, a moderate inhibitory effect on Harding-Passey melanoma and Novikoff hepatoma, and a slight inhibitory effect on seven other tumors. Although antitumor activity of quinomycins A, B, and C was essentially the same qualitatively, quinomycin C appeared to be most active.

In the case of rat and hamster tumors, quinomycin B had essentially no inhibitory effect, whereas quinomycin A had a definite inhibitory effect. From these results, it appears that the type of N-methyl amino acid in the molecule influences the degree of activity.

TABLE 3. *Effect of the quinomycins and triostins on various mouse, rat, hamster, and chicken tumors*

| Drug | Quinomycin A | | Quinomycin B | | Quinomycin C | | Triostins | |
|---|---|---|---|---|---|---|---|---|
| *Mouse tumors* | Dose* | Evaluation | Dose | Evaluation | Dose | Evaluation | Dose | Evaluation |
| Sarcoma 180 (solid) | 0.05 | − | 0.01 | − | 0.01 | − | 25 | − |
| Sarcoma 180 (solid) | | | 0.005 | − | | | | |
| Sarcoma 180 (ascitic) | 0.05 | + | 0.005 | + | 0.01 | ++ | 25 | + |
| Sarcoma 180 (ascitic) | 0.025 | + | | | 0.005 | ++ | 12.5 | ± |
| Sarcoma T241 | 0.05 | − | 0.01 | − | 0.01 | − | 25 | − |
| Sarcoma T241 | | | 0.005 | − | 0.005 | − | 12.5 | − |
| Sarcoma MA387 | 0.05 | − | 0.005 | − | 0.01 | − | 25 | − |
| Sarcoma MA387 | | | | | 0.005 | − | | |
| Ehrlich carcinoma (solid) | 0.05 | − | 0.01 | − | 0.01 | ± | 25 | + |
| Ehrlich carcinoma (solid) | | | | | 0.005 | ± | | |
| Ehrlich carcinoma (ascitic) | 0.05 | + | 0.01 | + | 0.01 | +++ | 25 | ± |
| Ehrlich carcinoma (ascitic) | | | 0.005 | + | 0.005 | +++ | | |
| Bashford carcinoma 63 | 0.05 | − | 0.005 | − | 0.01 | − | 25 | − |
| Bashford carcinoma 63 | | | | | 0.005 | − | | |
| Adenocarcinoma E 0771 | 0.05 | − | 0.01 | − | 0.01 | − | 25 | ± |
| Adenocarcinoma E 0771 | | | 0.005 | − | | | | |
| Miyono adenocarcinoma | 0.05 | − | 0.005 | + | 0.005 | ± | 25 | + |
| Miyono adenocarcinoma | | | | | | | 12.5 | − |
| Carcinoma 1025 | 0.05 | − | 0.01 | − | 0.01 | − | 25 | + |
| Carcinoma 1025 | | | 0.005 | − | | | | |
| Lewis bladder carcinoma | 0.05 | − | 0.01 | − | 0.01 | − | 25 | + |
| Lewis bladder carcinoma | | | 0.005 | − | | | 12.5 | − |
| Lewis lung carcinoma | 0.05 | − | 0.005 | − | 0.01 | − | 25 | ± |
| Wagner osteogenic sarcoma | 0.05 | − | 0.01 | − | 0.01 | ± | 25 | − |
| Wagner osteogenic sarcoma | | | | | 0.005 | ± | 12.5 | − |
| Ridgway osteogenic sarcoma | 0.05 | − | 0.01 | − | 0.01 | − | 25 | + |
| Ridgway osteogenic sarcoma | | | 0.005 | − | | | 12.5 | + |
| Mecca lymphosarcoma | 0.05 | − | 0.01 | − | 0.01 | − | 25 | +++ |
| Mecca lymphosarcoma | | | | | | | 12.5 | + |
| Harding-Passey melanoma | 0.05 | − | 0.005 | ± | 0.01 | + | 25 | − |
| Harding-Passey melanoma | | | | | 0.005 | − | | |
| Glioma 26 | 0.05 | − | 0.01 | + | 0.01 | − | 25 | − |
| Friend virus leukemia | 0.05 | − | 0.005 | − | 0.005 | − | 25 | + |
| Friend virus leukemia | | | | | | | 12.5 | + |
| Friend virus leukemia (solid) | 0.05 | + | 0.005 | − | 0.005 | + | 12.5 | + |
| Leukemia L 4946 | 0.05 | − | 0.01 | − | 0.01 | − | 25 | − |
| Leukemia L 4946 | | | | | 0.005 | − | | |
| *Rat tumors* | | | | | | | | |
| Flexner-Jobling carcinoma | 0.025 | − | 0.005 | − | 0.005 | − | 1.0 | − |
| Walker carcinosarcoma 256 | 0.025 | ± | 0.01 | − | 0.005 | − | 1.0 | − |
| Walker carcinosarcoma 256 | | | 0.005 | − | 0.005 | − | | |
| Jensen sarcoma | 0.025 | + | 0.005 | − | 0.005 | − | 1.0 | − |
| Iglesias sarcoma | 0.025 | − | 0.005 | − | 0.005 | − | 1.0 | − |
| Moore sarcoma No. 1 | 0.025 | − | 0.005 | − | 0.005 | − | 1.0 | − |
| Murphy-Sturm lymphosarcoma | 0.025 | + | 0.005 | − | 0.005 | + | 1.0 | − |
| Murphy-Sturm lymphosarcoma | 0.0125 | ± | | | | | | |
| Iglesias ovarian tumor | | | 0.005 | − | 0.005 | − | 1.0 | − |
| Iglesias adrenal tumor | 0.025 | − | | | 0.0025 | − | 1.0 | − |
| Babcock kidney tumor | 0.025 | | 0.01 | ± | 0.005 | + | 1.0 | − |
| Babcock kidney tumor | | | 0.005 | − | | | | |
| Novikoff hepatoma | 0.025 | − | 0.005 | − | 0.005 | + | 1.0 | − |
| *Hamster tumors* | | | | | | | | |
| Crabb sarcoma | 0.05 | ± | 0.0025 | − | 0.005 | − | 1.0 | − |
| Crabb sarcoma | 0.025 | − | | | | | | |
| Fortner pancreatic tumor | 0.05 | ± | 0.005 | − | 0.005 | − | 1.0 | − |
| Fortner pancreatic tumor | 0.025 | − | | | | | | |
| Fortner small intestine tumor | 0.025 | + | 0.005 | − | 0.005 | ± | 1.0 | − |
| Fortner small intestine tumor | | | 0.0025 | − | | | | |
| *Chicken tumor* | | | | | | | | |
| Rous sarcoma | 0.025 | − | 0.01 | − | 0.01 | − | 1.0 | − |

*Daily dose (mg/kg).

The triostins had different activity against the tumors than did the quinomycins and affected the growth of more mouse tumors. The triostin mixture was strongly active against Miyono adenocarcinoma, carcinoma 1025, Ridgway osteogenic sarcoma, Mecca lymphosarcoma, and Friend virus leukemia, whereas only the Miyono adenocarcinoma responded to the quinomycins. Therefore, the N,N-dimethylcystine in the structure of triostin apparently plays an important role in its biological activity.

Quinomycin B and the triostins, all at maximal tolerated dose levels, were less effective against rat, hamster, and chicken tumors than they were against mouse tumors. Quinomycin A, on the other hand, was effective against rat and hamster tumors. It had a moderate inhibitory effect on Jensen sarcoma and a slight inhibitory effect on five other tumors.

## Discussion

One of our particular interests in the screening program was to learn whether any antibiotics structurally related to the actinomycins have antitumor activity. Therefore, we studied the quinoxaline antibiotics.

Quinoxaline antibiotics are included, as are the actinomycins, in the category of chromopeptide antibiotics. Both antibiotics inhibit mainly gram-positive bacteria and show similar cytotoxicity to tissue culture cells.

It was noted that an actinomycin C-resistant strain of *Sarcina lutea* showed cross-resistance to quinoxaline antibiotics.

The minimal inhibitory concentration against certain bacteria, the $LD_{50}$ in mice, and the tumor spectrum of many actinomycins (about 35 are known) are similar (Katz and Pugh, 1961; Sugiura, 1960). On the other hand, several quinoxaline antibiotics showed various differences in the above-mentioned characteristics.

Comparison of the results with the Russian antibiotic 6270 are interesting. Although the description of the physicochemical properties is not too clear, this antibiotic seems to be similar to quinomycin C or B (Ivantiskaya et al., 1961). Antibiotic 6270 caused atrophy of the spleen. It was found to be more effective against lymphosarcoma than against sarcoma 180 or Ehrlich carcinoma (Rossolimo, Stanislavskaya, and Lepeshkina, 1959). Quinomycins, however, have no activity against our strain of lymphosarcoma (Mecca lymphosarcoma) and do not cause atrophy of the spleen in mice.

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## Literature Cited

Carter, H. E., C. P. Schaffner, and D. Gottlieb. 1954. Levomycin. I. Isolation and chemical studies. Arch. Biochem. Biophys. **53**:282-293

Ivantiskaya, L. P., E. B. Kruglyak, T. S. Maksimova, and T. P. Preobrazhenskaya. 1961. The production of echinomycin-like substances by various species of actinomycetes. Antibiotiki **6**: 393-397.

Katz, E., and L. H. Pugh. 1961. Variation on theme. The actinomycins. Appl. Microbiol. **9**:263-267.

Keller-Schierlein, W., M. L. Mihailvic, and V. Prelog. 1959. Stoffwechsel Produkte von Actinomyceten. Über die Konstituten von Echinomycin. Helv. Chim. Acta **42**:305-322.

Kuroya, M., N. Ishida, K. Katagiri, J. Shoji, T. Yoshida, M. Mayama, K. Sato, S. Matsuura, Y. Niinome, and O. Shiratori. 1961. Studies on quinoxaline antibiotics. I. General properties and the producing strains. J. Antibiotics (Japan) **13A**:324-329.

Preobrazhenskaya, T. P., L. P. Ivanitskaya, E. G. Toropova, G. F. Gauze, N. P. Ilicheva, G. V. Gavrilina, and K. K. Ivanov. 1958. Cultural conditions for the formation of the cancer-inhibiting antibiotic 6270. Puti i Metody Izyskan, Protivorak. Antibiotikov, Trudy Simoziuma, Moscow, p. 101-109 (Chem. Abstr. **54**:25028e, 1960).

Rossolimo, O. K., M. S. Stanislavskaya, and G. N. Lepeshkina. 1959. Experimental study of the antitumor substances of the new antibiotic 6270. Antibiotiki **4**:54-59.

Shoji, J., and K. Katagiri. 1961. Studies on quinoxaline antibiotics. III. New antibiotics, triostins A, B, and C. J. Antibiotics (Japan) **14A**:335-339.

Sugiura, K. 1953. Effect of various compounds on the Ehrlich ascites carcinoma. Cancer Research **13**:431-441.

Sugiura, K. 1959. Effects of compounds on the Friend mouse virus leukemia. Gann **50**:251-264.

Sugiura, K. 1960. The effect of actinomycin D on a spectrum of tumors. Ann. N. Y. Acad. Sci. **89**: 368-372.

Sugiura, K., and C. C. Stock. 1952. Studies in a tumor spectrum. I. Comparison of the action of methyl bis (2-chloroethyl) amine and 3-bis (2-chloroethyl) aminomethyl-4-methoxymethyl-5-hydroxy-6-methyl pyridine on the growth of a variety of mouse and rat tumors. Cancer **5**: 382-402.

Ueda, M., Y. Tanigawa, Y. Okami, and H. Umezawa. 1954. A new toxic, actinoleukin, produced by a streptomycete. J. Antibiotics (Japan) **7**:125-126.

Yoshida, T., and K. Katagiri. 1961. Studies on quinoxaline antibiotics. II. Isolation and properties of quinomycins A, B, and C. J. Antibiotics (Japan) **14**:330-334.

# PRODUCTION OF A NOVEL CYTOTOXIC AGENT, SQ 15,859, BY *STREPTOMYCES CHRYSOMALLUS*

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In the course of a survey of microorganisms for the production of agents inhibiting the growth of tumors in mice, an extract from a streptomycete, isolated from a soil sample, showed activity against sarcoma 180. This streptomycete was identified as *Streptomyces chrysomallus* on the basis of its morphological and physiological characteristics. Upon fractionation of the fermented media (Dutcher, 1961), two cytotoxic materials were isolated and crystallized. One was identified as actinomycin C; the other was designated as SQ 15,859. SQ 15,859 has been found to have cytotoxic, antitumor, and antibacterial activities. We shall summarize some of our observations in this communication.

## Materials and Methods

*Fermentation.* Flasks of a medium containing soybean meal (solvent extraction process), dehydrated mashed potatoes, glucose, calcium carbonate, agar, cobalt chloride, and tap water were inoculated with spores of the streptomycete and incubated at 25 C on a rotary shaker. After 72 hr incubation the vegetative growth was used to inoculate a second series of Erlenmeyer flasks (125 ml medium per 500-ml flask), which were incubated on the rotary shaker for 144 to 168 hr. At the end of this incubation period, the contents of the flasks were pooled and the SQ 15,859 recovered by procedures described by Dutcher (1961).

## Results

*Distribution of streptomycetes producing SQ 15,859.* Two strains of *S. chrysomallus* (designated as SC 2846 and SC 3474) were found to produce actinomycin C and SQ 15,859. Examination of the fermentation liquors of the actinomycin producers, *S. antibioticus* (ATCC 8663), *S. flavus* (ATCC 3369), and *S. parvullus* (ATCC 12434), showed

that they did not produce SQ 15,859. It seems likely that the mechanisms of biosynthesis of actinomycins and SQ 15,859 do not always occur together in streptomycetes.

*Antibacterial spectrum of SQ 15,859.* A chloroform solution of SQ 15,859 was used in experiments to study the antibacterial spectrum of the material as determined by the agar dilution procedure (Table 1). The material was essentially inactive, although there was some inhibition of gram-positive organisms when the concentration was 250 μg per ml of agar.

TABLE 1. *Antibacterial spectrum of SQ 15,859*

| Organism | Minimal inhibitory concentration | |
|---|---|---|
| | In agar | In broth |
| | μg/ml | μg/ml |
| *Aerobacter aerogenes* | >1,000 | >10 |
| *Bacillus cereus* | 250 to 1,000 | 2 |
| *Corynebacterium simplex* | 500 | 2.7 |
| *Escherichia coli* | >1,000 | >33 |
| *Klebsiella pneumoniae* | >1,000 | >10 |
| *Leuconostoc mesenteroides* | 250 | 2 |
| *Micrococcus lysodeikticus* | 125 to 250 | 0.01 |
| *Pseudomonas aeruginosa* | >1,000 | >8 |
| *Staphylococcus aureus* | 250 to 1,000 | 0.25 |

As broth dilution procedures are known to be more sensitive indicators of antibacterial activity than agar dilution or agar diffusion techniques, these methods were used. Samples of methanolic or ethanolic solutions of SQ 15,859 were added to Penassay broth (Difco) inoculated with various bacteria having cell densities of 1,000 to 2,000 cells per ml. Minimal inhibitory concentrations (as determined by visible growth after 24 hr incubation) are also summarized in Table 1. The antibacterial activity of SQ 15,859 apparently was affected by the insolubility of the material in aqueous solutions. The inoculum size, much larger in the agar dilution technique, may also have affected the results. Using the broth dilution procedure, a 1,000-fold increase in inoculum size produced a twofold increase in the minimal inhibitory concentration.

*Cytotoxic activity.* The cytotoxic activity of SQ 15,859 was tested against established cell lines grown in suspension culture by methods previously described (Perlman et al., 1959; Giuffre, Perlman, and Jackson, 1961). Among the cell lines used in these experiments were Earle's L cells (NCTC 929) and a cell line established from Ehrlich ascites tumors taken from a mouse (Jackson, Giuffre, and Perlman, 1960; Brindle et al., 1961). Cell densities were determined by counting samples in a Coulter Electronic Counter. The

concentrations of SQ 15,859 resulting in 50% inhibition of growth ($ID_{50}$) were determined graphically from dose-response curves.

When SQ 15,859 was added to Earle's L cells growing in Waymouth's medium MB 752/1 supplemented with methylcellulose (Giuffre et al., 1961), the $ID_{50}$ was 0.002 $\mu$g per ml. When the cells were grown in Waymouth's medium MB 752/1 supplemented with 10% (v/v) calf serum, the $ID_{50}$ was increased to 0.06 $\mu$g per ml. An $ID_{50}$ value of 0.1 $\mu$g per ml was obtained in experiments studying the inhibition of the Ehrlich ascites cell line growing in Waymouth's medium MB 752/1 supplemented with 10% (v/v) calf serum.

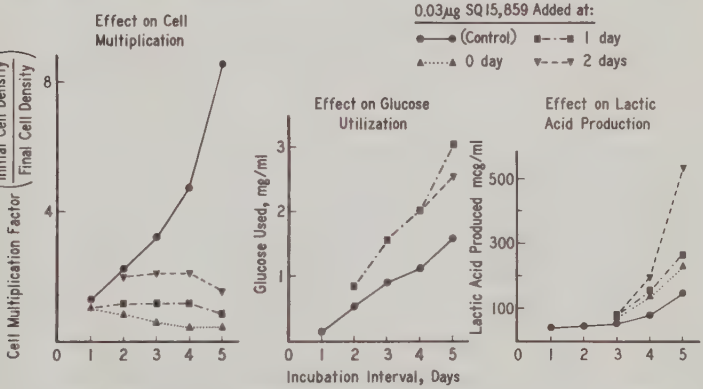


FIG. 1. *Effect of SQ 15,859 on growth and metabolism of Earle's L cells.*

Addition of SQ 15,859 to cultures of Earle's L cells growing in Waymouth's chemically defined medium MB 752/1 (supplemented with methylcellulose) resulted in increased acid production by the cultures (Fig. 1). Addition of the SQ 15,859, up to two days after initiation of growth of the cultures, resulted in inhibition of growth, increased synthesis of lactic acid, and some increase in the rate of glucose utilization. These effects on carbohydrate metabolism suggested that SQ 15,859 affected oxidative phosphorylation by the cells. Subsequent studies by Lardy (1961) showed SQ 15,859 to be a very effective phosphorylation uncoupler.

*Antitumor activity in animals.* Suspensions of SQ 15,859 implanted intraperitoneally were found to be nontoxic to mice. A chloroform solution of the compound diluted with sesame oil had an $LD_{50}$ of 45 mg per kg. When the compound formulated in this fashion was injected intraperitoneally into mice previously implanted with the Crocker Sarcoma 180, essentially no inhibition of growth of the tumor was found. However, significant antitumor activity was obtained when the formulation was injected subcutaneously (Table 2).

TABLE 2. *Effect of SQ 15,859 on growth of sarcoma 180 in mice*

| Dose | Drug* administered by intraperitoneal injection | | Drug* administered by subcutaneous injection | |
|---|---|---|---|---|
| | Survivors | Inhibition of tumor wt. | Survivors | Inhibition of tumor wt. |
| *mg/kg* | | % | | % |
| 2.5 | 6/6 | 4 | | |
| 5 | 6/6 | 12 | | |
| 10 | 5/6 | 10 | 6/6 | 18 |
| 20 | 5/6 | 17 | 5/6 | 19 |
| 30 | 3/6 | toxic | 6/6 | 20 |
| 40 | 0/6 | toxic | 6/6 | 14 |
| 50 | | | 6/6 | 51 |
| 60 | | | 6/6 | 50 |
| 70 | | | 5/6 | 74 |
| 80 | | | 5/5 | 68 |
| 100 | | | 4/5 | 74 |
| 150 | | | 3/6 | toxic |

*Formulated in sesame oil. The methods recommended by the Cancer Chemotherapy National Service Center (1959) were used in these tests.

Somewhat contradictory results have been obtained in experiments with the mammary adenocarcinoma CA 755. When the sesame oil formulation was injected subcutaneously, growth of the carcinoma was inhibited at a dose of 40 to 50 mg per kg in one experiment, although in another a dose of 60 mg per kg had no effect. It is apparent that the formulation and route of administration are problems which markedly effect the antitumor properties of this material.

## Acknowledgments

The authors express their appreciation to B. G. H. Thomas and his associates for the pharmacological data.

We are indebted to the contracting groups of the Cancer Chemotherapy National Service Center for the antitumor evaluations.

## Literature Cited

Brindle. S. A., N. A. Giuffre, B. J. Amrein, R. C. Millonig, and D. Perlman. 1962. Antibiotic sensitivity of Ehrlich ascites cells grown in tissue culture. Antimicrobial Agents and Chemotherapy - 1961, p. 159-161.

Dutcher, J. D. 1962. Isolation and characterization of a cytotoxic agent, SQ 15,859, from *Streptomyces chrysomallus*. Antimicrobial Agents and Chemotherapy - 1961, p. 173-177.

Giuffre, N. A., D. Perlman, and P. W. Jackson. 1961. Effect of medium composition on sensitivity in suspension culture of Earle's L-929 mouse fibroblasts to antibiotics. Cancer Chemotherapy Reports **11**:57-60.

Jackson, P. W., N. A. Giuffre, and D. Perlman. 1960. Growth of Ehrlich ascites tumor cells in tissue culture. Can. J. Biochem. Physiol. **38**:1377-1378.

Lardy, H. A. 1961. Antibiotics as tools for studying oxidative phosphorylation. Abstr. Papers Am. Chem. Soc., p. 29C, 140th Meeting.

Perlman, D., N. A. Giuffre, P. W. Jackson, and F. E. Giardinello. 1959. Effects of antibiotics on multiplication of L cells in suspension culture. Proc. Soc. Exptl. Biol. Med. **102**:290-292.

# ISOLATION AND CHARACTERIZATION OF A CYTOTOXIC AGENT, SQ 15,859, FROM *STREPTOMYCES CHRYSOMALLUS*

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This investigation was undertaken to describe the activities of an antibacterial and cytotoxic agent produced by *Streptomyces chrysomallus*. In view of the marked biological activity of this product, SQ 15,859 (Bennett et al., 1962), we were interested in further characterizing its chemical and physical properties and, if possible, in elucidating its organic structure.

## Materials and Methods

SQ 15,859 has been isolated from whole culture broth and also, for convenience, from the mycelial cake. The mycelial cake has been found to contain the bulk of the substance present in the fermentation broth. When whole broth was taken, two volumes of *n*-propanol were added and the mixture agitated for 1 hr. The coagulated mycelium was separated by filtration or centrifugation and the filtrate concentrated in vacuo to remove the major portion of the propanol. The residual aqueous phase was repeatedly extracted with hexane and then with *n*-butanol. From the butanol residue, a material was isolated and identified as actinomycin C by its absorption spectrum and the paper chromatographic methods of Goss and Katz (1957). Evaporation of the combined hexane extracts yielded an oily residue which partially crystallized on standing. The crystalline material could be washed free of the oil with small portions of ether and then recrystallized from acetone or methanol. No trace of actinomycin could be detected in this purified material. An average yield of 35 to 50 mg of SQ 15,859 per 150 ml of culture broth was obtained.

*Physical properties.* Solubility: very soluble in chloroform, pyridine, *N,N*-dimethylformamide, and benzene; moderately soluble in acetic acid, tetrahydrofuran, and acetone; limited solubility

in methanol, ethanol, and ether; insoluble in water (no effect of pH). Melting point: 148 to 152 C (capillary, uncorrected). Ultraviolet absorption: no bands above 215 mμ. Infrared: strong bands at 5.8, 7.5, 7.9, and 8.4 μ. Stability: can be crystallized from warm glacial acetic acid; not attacked by 0.1 N methanolic KOH at 24 C; and slowly hydrolyzed at reflux temperature.

*Chemical properties.* Analysis: C, 65.3% (C-methyl, 15.0%); H, 8.5%; O (by difference), 26.2%. Salt: crystalline perchlorate; Cl, 4.5%; neutralization equivalent, 839. Molecular weight: by vapor-pressure lowering, 700. Empirical formula: $C_{40}H_{64}O_{12}$. Saponification equivalent: ethanolic KOH, 184. Tests: no acidic groups; no methoxyl; no acetyl; no acetylatable hydroxyl; no consumption of periodate; no uptake of $H_2$ with Pt in acetic acid; negative Brady, Molisch, and $FeCl_3$ tests.

As may be seen from these data, SQ 15,859 is a remarkably stable substance and rather inert chemically. It is a neutral compound, containing only carbon, hydrogen, and oxygen atoms, with no demonstrable functional groups such as hydroxyl or ketonic carbonyl, and it is not unsaturated as shown by its indifference to hydrogenation in the presence of platinum catalyst. Various methods for determining its molecular weight were applied, with results which varied from 500 to 700. Perhaps the most satisfactory clue to the empirical composition, $C_{40}H_{64}O_{12}$, is that furnished by the perchloric acid salt. This nicely crystalline product is formed when a few drops of 60% perchloric acid are added to a glacial acetic acid solution of SQ 15,859. A product of constant composition is formed regardless of the ratio of acid to compound. The perchlorate, which must be an oxonium salt, can be recrystallized from acetic acid but is readily decomposed with water or alcohol to regenerate the neutral SQ 15,859.

The analytical value of chlorine, 4.5%, and the neutralization equivalent of 839 are in agreement with the formulation of this product as $C_{40}H_{64}O_{12}\cdot HClO_4$. This formation of an oxonium salt is confirmative evidence for the conclusion that four of the oxygen atoms in SQ 15,859 must be present as ether functions. A strong band in the infrared absorption spectrum at 5.8 μ indicated that ester groupings were also present. To confirm this, the hydrolysis of SQ 15,859 with alkali was investigated. At 24 C in 0.5 N methanolic potassium hydroxide, the compound remained unchanged, but at the reflux temperature of 0.5 N ethanolic potassium hydroxide (ca. 80 C) saponification was complete in 6 hr. The saponification equivalent of 184 showed that 4 moles of alkali per mole of SQ 15,859 ($C_{40}H_{64}O_{12}$) were consumed. The acidic saponification product accounted for the entire weight of material and could be obtained in crystalline form as its barium or benzhydrylamine salt. Analytical and chemical characterization of this product showed it to be a hydroxy acid of the composition $C_{10}H_{18}O_4$. Thus, SQ 15,859 appears to be a cyclic ester composed of four $C_{10}H_{18}O_4$ units.

The position of the hydroxyl group in the hydroxy acid is such that the latter does not readily lactonize, even when treated with acetic anhydride and pyridine. The hydroxyl group has been shown to be secondary by oxidation of the hydroxy acid to a keto acid with chromium trioxide. This keto acid yielded iodoform in a qualitative test with hypoiodite, indicating the presence of a methyl ketone grouping, but a preparative oxidative degradation with this reagent could not be achieved and a reservation was held for a time as to whether or not such a grouping was actually present. However, an examination of the nuclear-magnetic-resonance spectrum of the methyl ester of the keto acid revealed unequivocally that the grouping $CH_3-\overset{|}{C}=O$ was present. The NMR spectra of SQ 15,859 itself and the acetylated methyl ester of the hydroxy acid also confirmed the presence in them of the grouping $CH_3-\overset{|}{\underset{|}{C}}-O-$.
H

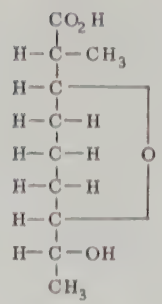
Additional confirmation of the ester groupings was obtained by lithium aluminum hydride reduction of SQ 15,859. This reaction led to the formation of a homogeneous, neutral product, $C_{10}H_{20}O_3$, which showed no carbonyl absorption bands in the infrared absorption spectrum.

## Results and Discussion

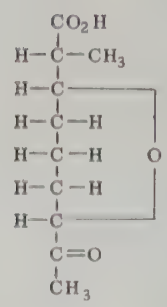
Although, to date, the complete structure of the $C_{10}$ hydroxy acid and of SQ 15,859 has not been elucidated, speculation as to a tentative structure is possible. The presence of a branched carbon chain is indicated by the analytical finding of two C-methyl groups in the $C_{10}$ unit. By considering all the chemical and physical data presently available, the placement of the functional groups can best be made in the following manner. If formula 1 represents the carbon skeleton, then by considering $C_1$ as the carboxyl group, $C_2$, the $\alpha$-carbon, would bear a branching methyl group. Such a formulation would be in line with the moderate degree of resistance to saponification observed for the ester linkages of SQ 15,859. The location of the secondary alcohol grouping must then be at $C_8$ as shown in formula 2, in view of the formation of a methyl ketone grouping on oxidation, formula 3. The size and location of the cyclic ether cannot be stated with certainty as yet, although the tetrahydropyran structure shown in formulas 2 and 3 is in best accord with the proton placements found in the NMR spectra.

(1)

(2)

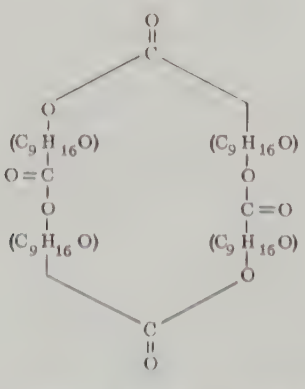


(3)



The structure of the SQ 15,859 molecule could be represented schematically as shown in formula 4. Aside from requiring further degradation to confirm or refute these suggested structures, it may be seen that the $C_{10}$ unit contains four asymmetric carbon atoms, and considerable work will be required to establish their configurations.

(4)



In surveying the literature for reports of similar products, the paper by Corbaz et al. (1955) was found in which these authors record the properties of an actinomycete metabolic product which they named nonactin. Its physical and chemical properties are quite similar to those of SQ 15,859. Through the kindness of V. Prelog we obtained a sample of nonactin for direct comparison. No marked differences in the infrared absorption spectrum could be seen and only minor differences in the melting points were detected. However, there were considerable differences in the biological activities of the two products.

Among the known antibiotics with cytotoxic and antitumor activity, SQ 15,859 thus represents a rather novel and unique structure.

### Acknowledgment

The author wishes to acknowledge the assistance of Jon H. Sherman and John A. Kowald in the experimental work, and A. Cohen of the Applied Physics Division, The Squibb Institute for Medical Research, for the preparation and interpretation of the nuclear-magnetic-resonance spectral data.

### Literature Cited

Bennett, R. E., S. A. Brindle, N. A. Giuffre, P. W. Jackson, J. Kowald, F. E. Pansy, D. Perlman, and W. H. Trejo. 1962. Production of a novel cytotoxic agent, SQ 15,859, by *Streptomyces chrysomallus*. Antimicrobial Agents and Chemotherapy - 1961, p. 169-172.

Corbaz, R., L. Ettlinger, E. Gäumann, W. Keller-Schierlein, F. Kradolfer, L. Neipp, V. Prelog, and H. Zähner. 1955. Stoffwechsel Produkte von Actinomyceten. 3 Mitteilung, Nonactin. Helv. Chim. Acta 38:1445-1448.

Goss, W. A., and E. Katz. 1957. Actinomycin formation by *Streptomyces* cultures. Appl. Microbiol. 5:95-102.

# CHEMISTRY OF THE DUAZOMYCINS

## I. Duazomycins A

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Duazomycins A, B, and C (same as diazomycins A, B, and C) are a group of antitumor substances produced by a culture of *Streptomyces ambofaciens* (Rao et al., 1960). They exhibit a strong inhibitory effect on sarcoma 180 and carcinoma 755 in mice. Their general chemical properties classify them in the small but unique group of aliphatic diazoketone-type compounds isolated from natural sources. In this respect they bear a close resemblance to the known antitumor compound 6-diazo-5-oxo-L-norleucine which also accompanies the duazomycins in the culture filtrate of *S. ambofaciens* (Rao et al., 1960; Dion et al., 1956). Duazomycin A is the major component of the three and the determination of its structure forms the subject matter of this paper.

### Materials and Methods

Duazomycin A is purified and crystallized as the lithium salt. Figures 1 and 2 show the ultraviolet and infrared spectra of this

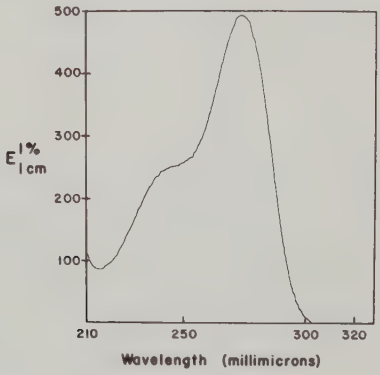


FIG. 1. *Ultraviolet spectrum of duazomycin A.*

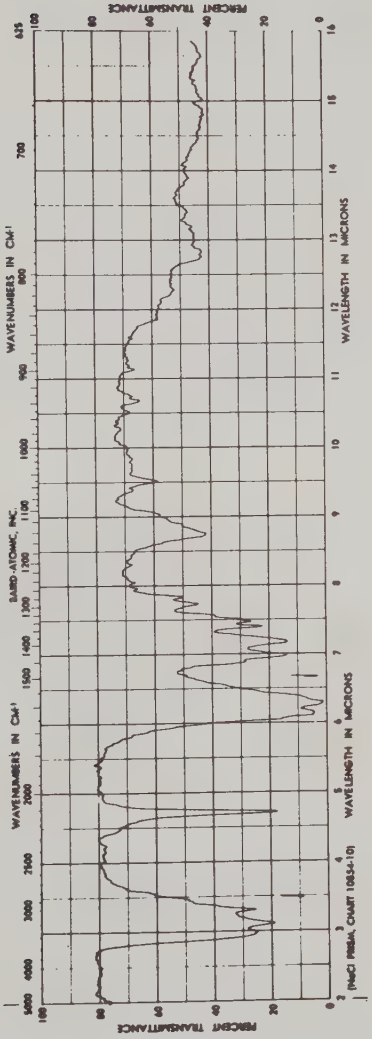


FIG. 2. *Infrared spectrum of duazomycin A.*

salt. The ultraviolet spectrum is typical of an aliphatic diazoketone and, as expected, is completely destroyed on adding acid. The significant infrared bands are the strong absorption band at 4.7 $\mu$, characteristic of the aliphatic diazo group, and intense multiple bands in the carbonyl region. The lithium salt of duazomycin A is very soluble in water and methanol and moderately soluble in ethanol. Concentrated solutions in methanol and ethanol tend to form thick gels readily.

Elemental analysis indicated an empirical formula of $C_8H_{11}O_4N_3$ for the metal-free duazomycin A. Determination of nitrogen by the Dumas and Kjeldahl methods showed that one-third of the nitrogen is acid-stable. Among the group analyses, the most significant one was the presence of one C-methyl group. Titration of the lithium salt with acid (Fig. 3A) showed that the compound is a moderately strong acid ($pK_a = 3.5 \pm 0.3$) with an equivalent weight of $220 \pm 10$, which is in agreement with the empirical formula. Duazomycin A gave a negative biuret test and a grayish-blue, feebly positive color reaction with ninhydrin. The compound did not react with 2,4-dinitrofluorobenzene under the conditions used in the case of amino acids. It was also not absorbed by strong acid-type cation-exchange resins such as Amberlite IR-120 or Dowex-50, although these resins readily decomposed the compound.

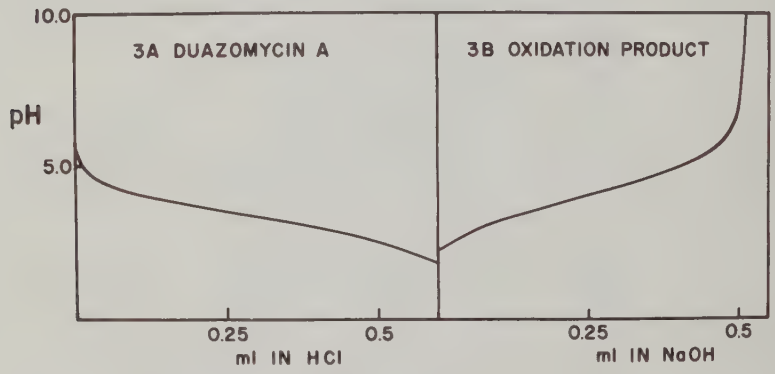


FIG. 3. *Titration of duazomycin A and its oxidation product.*

Direct acid hydrolysis of duazomycin A by refluxing with 6 N HCl resulted in extensive tar formation, presumably due to the presence of the keto group and an amino group in the molecule, and attempts at isolation of the products were not productive.

Duazomycin A readily reacts with periodic acid in a ratio of one mole per unit of $C_8H_{11}O_4N_3$. When the reaction mixture is

distilled, the distillate contains formaldehyde which arises from the hydrolysis and oxidation of the diazoketo group.

The second product of the oxidation was isolated as a colorless crystalline solid after the removal of the iodate and the unused periodate. The product was strongly acidic and titration (Fig. 3B) showed an equivalent weight of 95 ± 5. Elemental analysis gave an empirical formula of $C_7H_{11}O_5N$. The compound still did not give any significant color test with ninhydrin reagent. Acid hydrolysis of the oxidation product proceeded smoothly, in contrast to that of duazomycin A, and the hydrolysis mixture gave a strongly positive ninhydrin color. Paper chromatography at this stage revealed glutamic acid as the only ninhydrin-positive component. This compound was isolated and shown to be glutamic acid by analysis and infrared spectra.

These results and the presence of the C-methyl group suggested that the periodate-oxidation product might be N-acetyl glutamic acid. This was proved to be correct by direct comparison with an authentic sample by infrared spectra (Fig. 4). Based on the above results, duazomycin A is assigned the structure as N-acetyl 6-diazo-5-oxonorleucine.

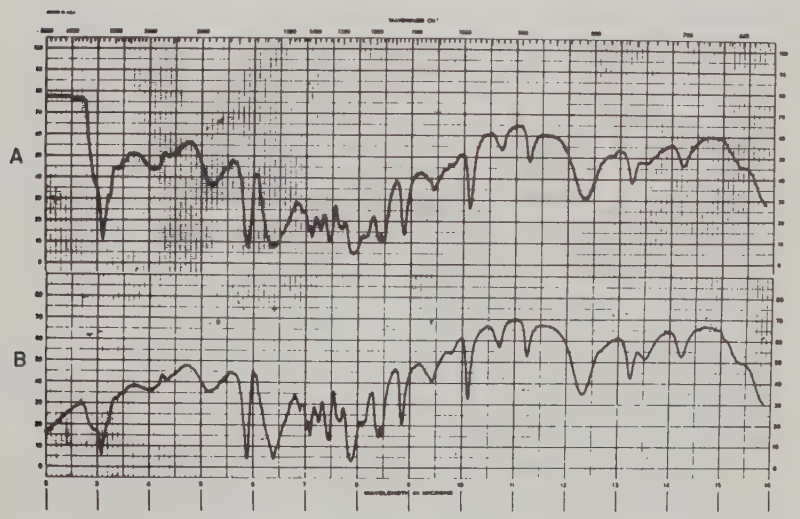


FIG. 4. *Infrared spectra of the oxidation product of duazomycin A* (A) *and of N-acetyl glutamic acid* (B).

## Experimental

Duazomycin A is purified by chromatography on Dowex-1 acetate resin. The crude material, mostly duazomycin A (5 g in 50 ml water), was added to a column of Dowex-1 acetate (200 ml of wet volume). After washing with water the column is eluted with 0.3% lithium phosphate buffer (pH 7.0 to 7.5). The progress of the column was followed by optical density at 275 mμ and paper chromatography in the system of isopropanol-water (4:1). The appropriate fractions were combined and freeze-dried. The solid was digested with ethanol and filtered. Concentration of the filtrate to a small volume resulted in a pale-yellow, thick gel which was broken up, filtered, and dried in a vacuum desiccator. The sample absorbs moisture from air and becomes crystalline.

*Analysis.* Found: C, 43.40%; H, 4.97%; $N_D$, 18.66%; $N_K$, 6.30%; Li, 2.64%; and C-$CH_3$, 6.67%. Calculated for $C_8H_{10}O_4N_3Li$: C, 43.86%; H, 4.60%; $N_D$, 19.16%; $N_K$, 6.38%; Li, 3.17%; and C-$CH_3$, 6.80%.

*Oxidation.* Duazomycin A (1 g) was treated with periodic acid (0.5 N, 20 ml). A current of nitrogen was passed through the reaction mixture and the gas bubbled through a solution of 2,4-dinitrophenylhydrazine in 2 N HCl. After 20 hr, titration of a sample revealed that the reaction was complete. The reaction mixture was freed from the iodate and unused periodate by the following procedure. Addition of a slight excess of hydriodic acid decomposed both iodate and periodate to produce iodine, which was filtered off. The hydriodic acid in the filtrate was oxidized to iodine by adding a slight excess of hydrogen peroxide. After complete removal of the resulting iodine by extraction with chloroform, the excess hydrogen peroxide was decomposed to water by platinum black. The final filtrate was then freeze-dried. The product was crystallized from a mixture of methanol and ethanol. It separated out as colorless rectangular prisms.

*Analysis.* Found: C, 45.03%; H, 6.07%; and N, 7.36%. Calculated for $C_7H_{11}O_5N$: C, 44.44%; H, 5.86%; N, 7.41%.

The 2,4-dinitrophenylhydrazone which separated out in the trap was filtered and crystallized from aqueous methanol. The product melted at 165 to 166 C alone or in admixture with the 2,4-dinitrophenylhydrazone of formaldehyde.

The oxidation product from the periodic acid reaction (0.5 g) was refluxed with 6 N HCl for 6 hr. The reaction mixture was concentrated to dryness and the product crystallized from methanol. It separated as colorless needles.

*Analysis.* Found: C, 41.20%; H, 6.34%; and N, 9.22%. Calculated for $C_5H_9O_4N$: C, 40.81%; H, 6.17%; and N, 9.52%. Its infrared spectrum was identical with that of glutamic acid.

## Literature Cited

Dion, H. W., S. A. Fusar, S. L. Jakubowski, J. G. Zora, and O. R. Bartz. 1956. 6-Diazo-5-oxonorleucine, a new tumor inhibitory substance. II. Isolation and characterization. J. Am. Chem. Soc. **78**:3075-3076.

Rao, K. V., S. C. Brooks, M. Kugelman, and A. A. Romano. Diazomycins A, B, and C, three antitumor substances. I. Isolation and characterization. Antibiotics Ann. 1959-60, p. 943-949.

# PACTAMYCIN, A NEW ANTITUMOR ANTIBIOTIC

## I. Discovery and Biological Properties

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*Research Laboratories, The Upjohn Company, Kalamazoo, Michigan*

Pactamycin (NSC #52947) is a new antibiotic produced by *Streptomyces pactum* var. *pactum*. Pactamycin is active in vitro against KB human epidermoid carcinoma cells in tissue culture and against a variety of gram-positive and gram-negative organisms. It is also active in vivo against a variety of tumors in mice and hamsters. This paper describes taxonomic studies, fermentation conditions necessary for production, and biological studies with the antibiotic. Other papers will describe the isolation and characterization studies (Jahnke, Argoudelis, and Fox, 1962) and the quantitative paper chromatogram method employed in the determination of pactamycin (Brodasky and Lummis, 1962).

### Materials and Methods

*Fermentation conditions*. The stock culture was a soil stock. Inoculum medium contained (g per liter): glucose monohydrate, 30; Pharmamedia, 40; pH 7.2, 48 hr incubation. The inoculum level was 5 ml per 100 ml medium. Sterilization was for 25 min at 121 C. The fermentation temperature was 32 C. A Gump rotary shaker was used, 250 rev/min; 2-inch stroke. The medium volume was 100 ml per 500-ml wide-mouth Erlenmeyer flask.

Taxonomic studies were based on Ektachrome comparisons (Dietz, 1954), carbon utilization studies (Pridham and Gottlieb, 1948), and microscopic appearance. Mycelial dry weight was determined by centrifuging 10 ml of broth, washing once with 5 ml of 0.1 N HCl, twice with water, and finally drying overnight at 100 C.

The activity in vitro against KB cells growing in tissue culture was determined by the method of Smith, Lummis, and Grady (1959). The activity in vitro against several bacteria was determined in Difco brain heart infusion broth, according to the method of Smith et al. (1956). Tissue culture chromatography methods are described by Brodasky and Lummis (1962).

## Results

*Taxonomy.* The culture has gray to bluish-gray aerial growth and gray to grayish-olive reverse on most media. Spores are borne in very compact spirals and were shown in electron micrographs to be covered with fine hairs (Fig. 1). The appearance of the culture

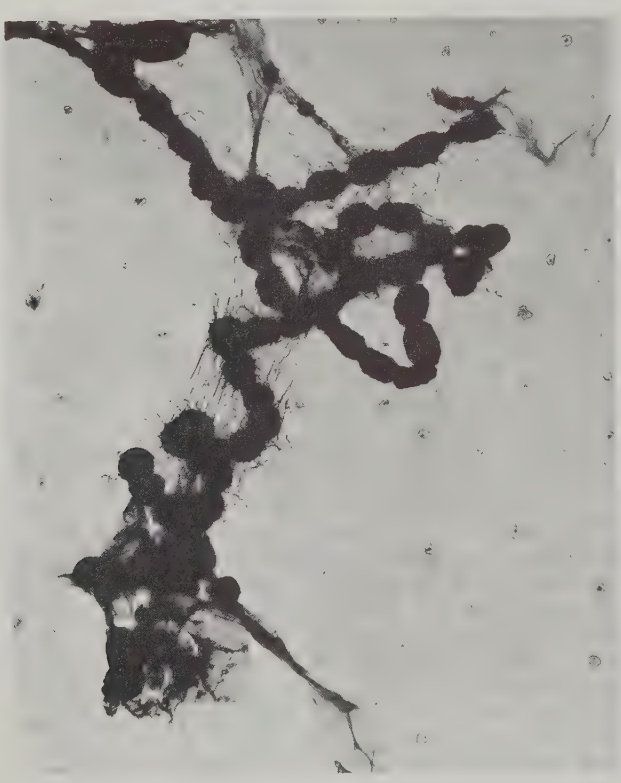

FIG. 1. *Electron micrograph of spores of S. pactum.*

on Ektachrome is shown in Table 1, the carbon assimilation pattern in Table 2, and macroscopic and physiological characteristics in Table 3. These studies indicated that the culture should be considered to be a new species for which the name *S. pactum* var. *pactum* is proposed. The culture has been assigned NRRL #2939.

*Paper chromatographic assay.* Since the components active against KB cells in agar were also active against *Bacillus subtilis*, the yield of pactamycin, the major component, was followed with a quantitative paper chromatographic assay using the bacterium.

TABLE 1. *Appearance of S. pactum on Ektachrome*

| Agar medium | Surface | Reverse |
|---|---|---|
| Bennett's | Gray-blue | Tan |
| Czapek's sucrose | Trace gray-white | Colorless |
| Maltose-tryptone | Gray-white | Yellow |
| Peptone-iron | None | Yellow |
| 0.1% Tyrosine | Trace gray-white | Pale yellow |
| Casein-starch | Gray-white | Pale yellow |

TABLE 2. *Assimilation of carbon compounds in chemically defined medium by S. pactum*

| Medium | Assimilation | Medium | Assimilation | Medium | Assimilation |
|---|---|---|---|---|---|
| D-Xylose | (+)[a] | Cellobiose | + | Salicin | (+) |
| L-Arabinose | (+) | Raffinose | (+) | Phenol | - |
| L-Rhamnose | (+) | Dextrin | + | Cresol | - |
| D-Fructose | (+) | Inulin | (+) | Na-formate | (+) |
| D-Galactose | + | Soluble starch | + | Na-oxalate | (+) |
| D-Glucose | + | Glycerol | + | Na-tartrate | - |
| D-Mannose | + | Dulcitol | (+) | Na-salicylate | - |
| Maltose | + | D-Mannitol | (+) | Na-acetate | + |
| Sucrose | (+) | D-Sorbitol | (+) | Na-citrate | (-) |
| Lactose | (+) | Inositol | (+) | Na-succinate | (-) |
| | | | | Control | (+) |

[a]Symbols: positive assimilation, +; positive assimilation, only slight growth, (+); slight growth, no assimilation, (-); no growth, -.

*Fermentation conditions.* Because pactamycin is bound to the mycelium in different proportions, depending on the fermentation conditions, an acetone extraction procedure was developed to determine the total amount of antibiotic produced in the fermentation. Antibiotic titer is expressed as total activity in the acetone extract.

The initial pH of all media was adjusted to 6.5 to 7.0. Since the maximum pactamycin yield was obtained at 32 C, all fermentations were conducted at this temperature.

Medium studies showed that good yields of pactamycin were obtained in a medium containing Cerelose (glucose monohydrate), Soy Peptone, calcium carbonate, and potassium chloride. The fermentation pattern obtained with this medium is shown in Fig. 2. Very little antibiotic production took place during the initial growth phase. Pactamycin was mainly produced during the later autolytic phase of the fermentation. The fermentation pH was about 8.3 during the entire production phase. A maximum titer of 216 μg pactamycin per ml was obtained after 5 days of fermentation.

*In vitro activity.* The in vitro activity of pactamycin against KB human epidermoid carcinoma cells and a variety of microorganisms

TABLE 3. *Macroscopic and physiological characteristics of S. pactum*

| Medium | Surface | Reverse | Other |
|---|---|---|---|
| Plain gelatin | Pale gray | - | Yellow pigment, partial liquefaction. |
| Nutrient gelatin | Gray-white | - | Yellow pigment, partial liquefaction. |
| Nutrient nitrate broth | - | - | Colorless vegetative growth dropping to base, yellow pigment, no reduction. |
| Synthetic nitrate broth | Pale gray-white | - | Surface pellicle, slight growth throughout, trace yellow pigment, reduction. |
| Litmus milk | Pale gray-white | Blue | Surface pellicle, peptonization, pH 7.1. |
| Peptone iron agar | Pale gray-pink | Yellow-tan | No $H_2S$ darkening. |
| Calcium malate agar | Pale gray-white | Pale gray-white | Malate not solubilized. |
| Skim milk agar | Pale gray-pink | Yellow | Yellow pigment, casein hydrolyzed. |
| Glucose asparagine agar | Pale lavender-pink | Pale yellow-tan | Pale yellow-tan pigment. |
| Tyrosine agar | Pale gray-white | Pale yellow | Pale yellow pigment, tyrosine solubilized. |
| Xanthine agar | Pale gray-white | Cream | Pale yellow pigment, slight xanthine solubilization under growth. |
| Maltose tryptone agar | Lavender-gray with cottony white tufts | Yellow-tan | No pigment. |
| Bennett's agar | Blue-gray | Yellow-tan | Yellow-tan pigment. Gray-white aerial mycelium at 18 and 24C, blue-gray at 28 and 37C, yellow reverse at 18 and 24C, yellow-tan at 28 and 37C, no growth at 55C. |
| Czapek's sucrose agar | Pale gray-white | Pale gray-white | No pigment, pale gray-white aerial mycelium at 18, 24, 28 C, gray at 37 C, yellow reverse at 18 and 24 C, gray-white at 28 C, gray-tan at 37 C, no growth at 55 C. |

is given in Table 4. Pactamycin was highly active against a human cancer cell in vitro and active primarily against gram-positive microorganisms. Pactamycin had marginal activity against a group of fungi, as shown in Table 5. Under these testing conditions, endomycin inhibited all these fungi at 1 to 10 $\mu$g per ml.

*In vivo activity of pactamycin.* Pactamycin was active against the tumors in mice and hamsters (Table 6). The antibiotic was inactive against the remaining tumors of the Sugiura spectrum (Sugiura, et al., 1958).

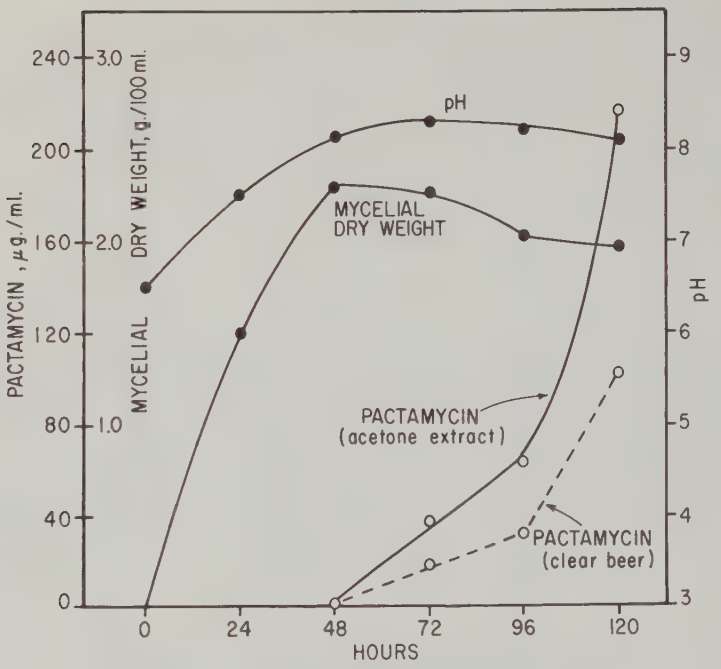


FIG. 2. *Cell weight, pH, and antibiotic courses during pactamycin production.*

TABLE 4. *In vitro activity of pactamycin*

| Test organism | Strain | $ID_{50}$ | MIC* |
|---|---|---|---|
| | | *μg/ml* | *μg/ml* |
| Mammalian cell | | | |
| KB - human epidermoid carcinoma | | 0.003 | |
| Bacteria | | | |
| *Bacillus subtilis* | UC-28 | | 0.8 |
| *Staphylococcus aureus* | OSU-284 | | 0.2 |
| *Staphylococcus aureus* (*albus* form) | ATCC 151 | | 0.05 |
| *Streptococcus pyogenes* | C 203 | | 1.6 |
| *Streptococcus faecalis* | ATCC 6057 | | 1.6 |
| Viridans group | UC 155 | | 3.2 |
| *Escherichia coli* | ATCC 26 | | 6.5 |
| *Klebsiella pneumoniae* | U. of Calif. A-D | | 0.8 |
| *Pasteurella multocida* | Lederle P-449 | | 0.012 |
| *Proteus vulgaris* | ATCC 6380 | | 110 |
| *Salmonella typhosa* | MSDH TG 3 | | 55 |
| *Salmonella pullorum* | 75 | | 1.6 |
| *Pseudomonas aeruginosa* | ATCC 9027 | | 110 |

*Minimal inhibitory concentration.

TABLE 5. *Antifungal activity of pactamycin*

| Fungi | Inhibition of growth[a] | |
|---|---|---|
| | 1,000μg/ml | 10μg/ml |
| *Nocardia asteroides* | + | - |
| *Blastomyces dermatitidis* | + | - |
| *Coccidioides immitis* | + | - |
| *Geotrichum* sp. | + | - |
| *Hormodendrum compactum* | + | - |
| *Phialophora verrucosa* | + | - |
| *Cryptococcus neoformans* | + | - |
| *Histoplasma capsulatum* | + | - |
| *Sporotrichum schenckii* | + | - |
| *Monosporium apiospermum* | + | - |
| *Trichophyton rubrum* | + | - |
| *Microsporum canis* | + | - |
| *Trichophyton interdigitale* | + | - |
| *Candida albicans* Abbott | + | - |
| *Trichophyton violaceum* | + | - |

[a]Indicates inhibition of growth, +; indicates no inhibition of growth, -.

TABLE 6. *Antitumor activity of pactamycin*

| Tumor | Investigator | Dose | Inhibition | Toxicity |
|---|---|---|---|---|
| | | *mg/kg* | % | |
| Ehrlich carcinoma | K. Sugiura[a] | 1 | 39 | ± |
| Glioma-26 | K. Sugiura | 1 | 35 | ± |
| Ca-755 | K. Sugiura | 2 | 30 | ± |
| Iglesias adrenal tumor | K. Sugiura | 1 | 47 | ± |
| Crabb hamster sarcoma | K. Sugiura | 0.5 | 7-39 | ± |
| Fortner adenocarcinoma of small bowel | K. Sugiura | 1 | 25 | + |
| Rous sarcoma | K. Sugiura | 0.5 | 30 | + |
| L-1210 G | J. Burchenal[a] | 2 | 40[d] | ? |
| Human melanoma (ME-1) in hamsters | A. Handler[b] | 1.5 | 58 | - |
| Human choriocarcinoma in hamsters | R. Hertz[c] | 0.75 | 90 | - |

[a]Sloan-Kettering Institute, New York.
[b]Childrens Cancer Research Foundation, Boston, Massachusetts.
[c]National Cancer Institute.
[d]Increase in survival time.

*Toxicity of pactamycin.* The single dose acute toxicity of pactamycin in mice and rats was determined by Wells Laboratories under Cancer Chemotherapy National Service Center (CCNSC) contract. The $LD_{50}$ in mice orally and intravenously was 10.7 mg per kg and 15.6 mg per kg, respectively. The intravenous $LD_{50}$ in rats was 1.4 mg per kg. Dogs were much more sensitive than mice and rats, the antibiotic being lethal to dogs at a single dose of 0.75 mg per kg. The dogs became extremely nervous and anorexigenic. Post-mortem examinations indicated ruptured liver and hyperemic intestines.

## Discussion

The original beer was inactive in the CCNSC three animal-tumor screen, namely, S-180, Ca-755, and L-1210. Pactamycin, detected for its cytotoxicity in tissue culture and purified by the use of a correlative *B. subtilis* assay, was found active against Ca-755, L-1210, and eight other animal tumors.

## Acknowledgments

This work was supported in part by contract SA-43-ph-1933, Cancer Chemotherapy National Service Center, National Cancer Institute, National Institutes of Health, Washington, D.C.

The authors are indebted to Bruce E. Bowersox for his excellent technical assistance. In vivo antitumor studies were performed by K. Sugiura, A. Handler, and R. Hertz. Acute toxicity studies were performed through the CCNSC.

## Literature Cited

Argoudelis, A. D., H. K. Jahnke, and J. Fox. 1962. Pactamycin, a new antitumor antibiotic. II. Isolation and characterization. Antimicrobial Agents and Chemotherapy - 1961, p. 191-197.

Brodasky, T. F., and W. L. Lummis. 1962. Pactamycin, a new antitumor antibiotic. III. Spectrophotometric quantitative paper chromatographic assay. Antimicrobial Agents and Chemotherapy - 1961, p. 198-204.

Dietz, A. 1954. Ektachrome transparencies as aids in actinomycete classification. Ann. N. Y. Acad. Sci. **60**:152-154.

Pridham, T. G., and D. Gottlieb. 1948. The utilization of carbon compounds by some Actinomycetales as an aid for species determination. J. Bacteriol. **56**:107-114.

Smith, C. G., W. L. Lummis, and J. E. Grady. 1959. An improved tissue culture assay. I. Methodology and cytotoxicity of antitumor agents. Cancer Research **19**:842-846.

Smith, C. G., A. Dietz, W. T. Sokolski, and G. M. Savage. 1956. Streptonivicin, a new antibiotic. I. Discovery and biologic studies. Antibiotics & Chemotherapy **6**:135-142.

Sugiura, K., C. C. Stock, H. C. Reilly, and M. M. Schmid. 1958. Studies in a tumor spectrum. VII. The effect of antibiotics on the growth of a mouse, rat and hamster tumor. Cancer Research **18**:66-77.

# PACTAMYCIN, A NEW ANTITUMOR ANTIBIOTIC

## II. Isolation and Characterization

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This paper concerns the isolation and purification of a new antibiotic, pactamycin, from a fermentation broth of *Streptomyces pactum*. Pactamycin is active in vitro against a variety of gram-positive and gram-negative microorganisms and inhibits the growth of KB epidermoid carcinoma cells in tissue culture. It is also active in vivo against several tumors in mice and hamsters. Chemical and physical properties of the material are described in this paper. The biological properties of pactamycin are described in a companion paper (Bhuyan, Dietz, and Smith, 1962).

### Materials and Methods

*Isolation.* Pactamycin has been isolated by the following procedure: The whole broth was acidified to pH 2.8 and filtered. The clear broth was cooled, adjusted to pH 8.5, and extracted with ethyl acetate. The antibiotic was transferred into water at pH 1.7. The aqueous solution was then extracted with ethyl acetate at pH 8.5. The ethyl acetate extracts were concentrated and purified by chromatography over Florisil. The antibiotic was eluted from the column with a mixture of Skellysolve B and acetone in the volume ratio 70:30. Pactamycin, obtained as a white amorphous powder by concentration of the eluates, was rechromatographed over Florisil.

Distribution of the twice-chromatographed material in the Craig counter current apparatus using a system consisting of cyclohexane, ethyl acetate, and water in the volume ratio 2:2:3 showed this material to be essentially pure. The isolation of pactamycin was accomplished by using *Bacillus subtilis* as the assay organism.

*Characterization.* Pactamycin is highly soluble in ethanol, chloroform, and methylene chloride. It is also soluble in benzene and ether, but insoluble in Skellysolve B and in cyclohexane. In water, the antibiotic is soluble in acidic ($pH < 5$) and basic ($pH > 9.5$) solutions. At the isoelectric point ($pH = 8.3$), it is insoluble.

Results of the elemental analyses of pactamycin, its hydrochloride, citrate, and oxalate, molecular weight determinations, and potentiometric titrations suggest the molecular formula $C_{28}H_{40}N_4O_8$.

The ultraviolet absorption spectrum (Fig. 1) in ethanol shows a strong absorption band at 239 mμ (a = 52.6) with a shoulder at 264 mμ (a = 14.7), and two weak absorption bands at 313 mμ (a = 5.2) and 356 mμ (a = 3.4). In 0.01 N ethanolic sulfuric acid, the maxima are at 238 mμ (a = 48), (sh) 262 mμ (a = 13.7), 314 mμ (a = 4.16), and 352 mμ (a = 3.0). In 0.01 N ethanolic potassium hydroxide the following maxima are observed: 238 mμ (a = 55), (sh) 264 mμ (a = 15.5), and 320 mμ (a = 5.94) (Fig. 2).

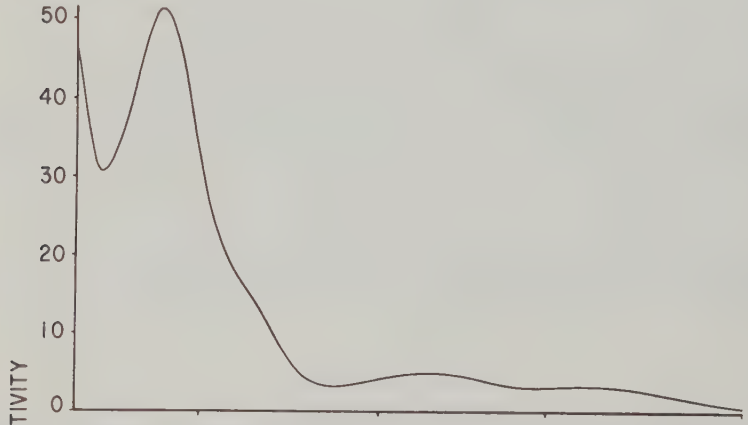


FIG. 1. *Ultraviolet spectrum of pactamycin in 95% aqueous ethanol.*

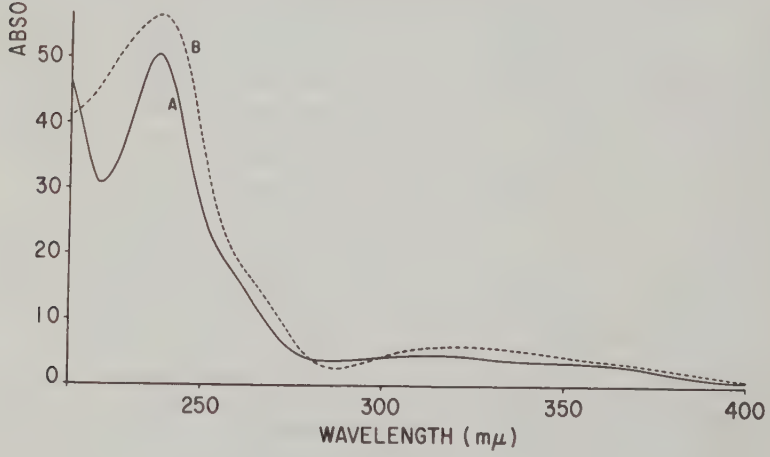


FIG. 2. *Ultraviolet spectrum of pactamycin in 0.01 N ethanolic sulfuric acid* (A) *and 0.01 N ethanolic potassium hydroxide* (B).

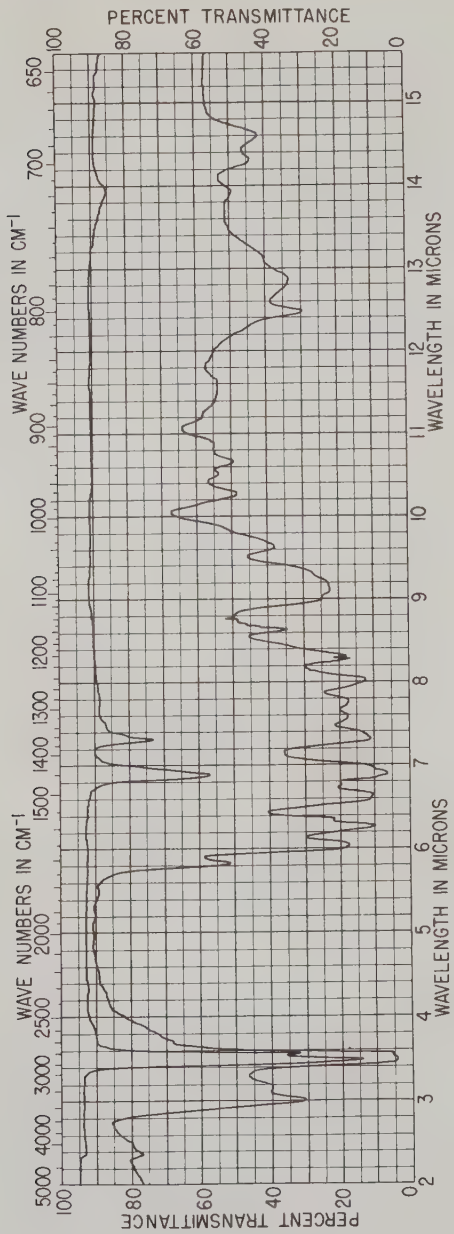


FIG. 3. *Infrared spectrum of pactamycin in mineral oil suspension. The upper curve represents mineral oil and the lower curve pactamycin.*

The infrared spectrum of the antibiotic in mineral oil suspension is shown in Fig. 3. The characteristic absorption bands are located at the following wave numbers: 3333, 3175, 1718, 1658, 1592, 1575, 1515, 1504, 1320, 1285, 1247, 1205, 1160, 1100, 1080, 1038, 975, 950, 940, 920, 870, 803, 778, 669, and 685 $cm^{-1}$.

The antibiotic gives a positive ninhydrin test but negative Molisch, Benedict, and ferric chloride tests.

Functional group analyses revealed the presence of one primary amino group (Van Slyke) and of four C-methyl groups (Kuhn-Roth).

Pactamycin is an optically active compound with a specific rotation $[\alpha]_D^{25}$ of + 79° (c = 1.0, 95% ethanol). On standing in aqueous or alcoholic solutions, the rotation changes to +23°. This change in the optical rotation does not cause an appreciable loss of bioactivity (Fig. 4).

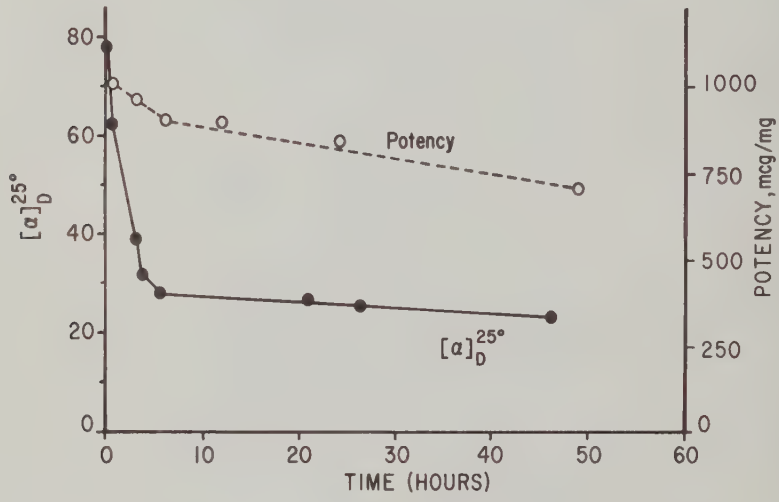


FIG. 4. *Specific rotation of pactamycin in 95% ethanol. Potency* (o) *and* $[\alpha]_D^{25}$ (•).

Pactamycin is an amphoteric substance. The basic function has a $pK'_a$ 7.25 (in water). The $pK'_a$ of the acidic function is 9.35. The equivalent weight was found to be 279.

Because of the presence of the basic function, salts of the antibiotic with acids can easily be prepared. The citrate, oxalate, and hydrochloride have been prepared as amorphous solids.

Pactamycin is rather unstable in solution. In 95% ethanol the antibiotic loses 20% of its biological activity after standing for 50 hr at room temperature. In an aqueous solution 25% of the bioactivity is lost in 24 hr at pH 7.

## Results

*Isolation from fermentation broth.* A culture broth (4,400 liters), containing pactamycin, was adjusted to pH 2.8 and heated to 60 C. The solution was cooled to 30 C, mixed with 176 kg of Dicalite 4200 (Great Lakes Carbon Co., Chicago, Ill.), and filtered. The clear broth was cooled to 5 C and adjusted to pH 8.5 by addition of 50% aqueous sodium hydroxide solution. The solution was then extracted three times with 1,450 liters of ethyl acetate. The first ethyl acetate extract was kept separate (Pool I) and the second and third extracts pooled (Pool II). Each pool was processed separately in the following manner: The ethyl acetate extracts were washed with water at pH 8.5, then extracted with deionized water at pH 1.7. The aqueous solution was adjusted to pH 8.5 and extracted twice with 4 liters of ethyl acetate. The final ethyl acetate extracts from Pools I and II were combined and concentrated to a volume of 2.6 liters containing 56 g of solids. This ethyl acetate concentrate was purified over Florisil as described below.

*Florisil chromatography.* Florisil (1,800 g) was poured slowly into a 3-inch diameter column filled with Skellysolve B. The adsorbent was allowed to settle under atmospheric pressure to a bed depth of 32 in. (holdup volume, 3,300 ml). The ethyl acetate concentrate obtained as described above was poured evenly on top of a 2-inch bed of sea-sand which had been placed on top of the Florisil bed. The column was then washed with one holdup volume of Skellysolve B-acetone mixtures of increasing acetone content. Fractions obtained by elution with Skellysolve B-acetone in the volume ratios of 75:25 and 70:30 were combined and concentrated to dryness to give 21 g of crude pactamycin. Then, 19 g of the crude antibiotic were dissolved in one liter of ether. Insoluble material was removed by filtration. The filtrate was dried over magnesium sulfate and concentrated to dryness. The residue was dissolved in 20 ml of methylene chloride and chromatographed over 300 g of Florisil packed in a 2-inch diameter column (holdup volume, 200 ml). The column was again eluted with Skellysolve B-acetone mixtures of increasing acetone content as presented in Table 1, and 10 200-ml fractions were collected.

Fractions 6, 7, 8, and 9 were combined and concentrated to dryness to give 8.4 g of a white colorless amorphous powder.

Analysis Calculated for $C_{28}H_{40}N_4O_8$; C, 60.06%; H, 7.20%, N, 9.99%. Molecular weight: 560. Found: C, 60.80%; H, 7.22%; N, 9.16%. Molecular weight: 531 (isothermal distillation).

Potentiometric titration revealed the presence of one basic and one acidic group with $pK'_a$ 7.25 and 9.35, respectively; equivalent weight, 279. U.V. [$\lambda$ maximum (a)] in ethanol: 239 m$\mu$ (51.22), (sh) 264 m$\mu$ (14.81), 313 m$\mu$ (4.88), and 356 m$\mu$ (3.25); $[\alpha]_D^{25}$ + 79° (c = 1.0, 95% ethanol).

TABLE 1. *Florisil chromatography of crude pactamycin*

| Fraction number | Eluting agent | Weight per fraction |
|---|---|---|
| | | *mg* |
| 1 | Skellysolve B | 200 |
| 2 | Skellysolve B-acetone (90:10) | 100 |
| 3 | Skellysolve B-acetone (80:20) | 100 |
| 4 | Skellysolve B-acetone (75:25) | 100 |
| 5 | Skellysolve B-acetone (70:30) | 200 |
| 6 | Skellysolve B-acetone (70:30) | 310 |
| 7 | Skellysolve B-acetone (70:30) | 5,200 |
| 8 | Skellysolve B-acetone (70:30) | 2,140 |
| 9 | Skellysolve B-acetone (70:30) | 780 |
| 10 | Skellysolve B-acetone (70:30) | 330 |

Counter current distribution of pactamycin. When 2 g of pactamycin, obtained as described above, were distributed in the system consisting of cyclohexane, ethyl acetate, and acetone in the volume ratio 2:2:3, the determination of solids and biological activity after 400 transfers revealed the presence of one peak (K = 5.0) which was in excellent agreement with the theoretical curve. Concentration of the peak tubes to an aqueous solution followed by lyophilization gave 1.7 g of a colorless amorphous solid.

Analysis Calculated for $C_{28}H_{40}N_4O_8$: C, 60.05%; H, 7.20%; N, 9.99%; O, 22.76%. Found: C, 60.33%; H, 7.07%; N, 9.34%; O, 22.99%. U.V. [λ max. (a)] in ethanol: 239 mμ (52.65), (sh) 264 mμ (14.76), 313 mμ (5.21), and 356 mμ (3.38); $[\alpha]_D^{25}$ + 22° (c = 1.0, 95% ethanol). The infrared spectrum (Fig. 3) was identical to that of the starting material.

Pactamycin hydrochloride. Gaseous hydrogen chloride was passed through a solution of 300 mg of pactamycin in 25 ml of ether. Pactamycin hydrochloride precipitated as a colorless amorphous solid and was purified by reprecipitation from methanol-ether. The yield was 325 mg.

Analysis Calculated for $C_{28}H_{40}N_4O_8 \cdot HCl$: C, 56.33%; H, 6.92%; N, 9.38%; O, 21.43%; Cl, 5.98%. Found: C, 56.44%; H, 6.79%; N, 8.84%; O, 21.57%; Cl, 6.32%; U.V. [λ max. (a)] in 95% ethanol, 238 mμ (46), (sh) 264 mμ (3.7), and 356 mμ (2.8); $[\alpha]_D^{25}$ + 30° (c = 0.6, 95% ethanol).

Pactamycin citrate. A saturated solution of citric acid in ether was added to a stirred solution of 500 mg of pactamycin in 25 ml of ether. Pactamycin citrate precipitated as an amorphous colorless solid (450 mg). This was purified by reprecipitation from acetone-ether.

Analysis Calculated for $C_{28}H_{40}N_4O_8 \cdot C_6H_8O_7$: C, 54.23%; H, 6.41%; N, 7.47%. Found: C, 54.53%; H, 6.74%; N, 7.25%. U.V. [λ max. (a)] in 95% ethanol, 238 mμ (37.7), (sh) 264 mμ (9.81), 312 mμ (3.3), and 356 mμ (2.3).

Pactamycin oxalate. A saturated solution of oxalic acid in ether was added to a solution of 300 mg of pactamycin in 25 ml of ether. Pactamycin oxalate precipitated as a colorless amorphous material (480 mg). It was purified by reprecipitation from methanol ether.

Analysis Calculated for $C_{28}H_{40}N_4O_8 \cdot C_2O_4H_2$: C, 55.43%; H, 6.51%; N, 8.62%. Found: C, 55.19%, H, 6.57%; N, 8.61%. $[\alpha]_D^{25} = 32^o$ (c = 0.7, 95% ethanol). U.V. [λ max. (a)] in 95% ethanol, 238 mμ (45.5), (sh) 264 mμ (12), 312 mμ (4.2), and 352 mμ (2.9).

## Acknowledgments

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## LITERATURE CITED

Bhuyan, B. K., A. Dietz, and C. G. Smith. 1962. Pactamycin, a new antitumor antibiotic. I. Discovery and biological properties. Antimicrobial Agents and Chemotherapy - 1961, p. 184-190.

# PACTAMYCIN, A NEW ANTITUMOR ANTIBIOTIC

## III. Spectrophotometric Quantitative Paper Chromatographic Assay

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A new cytotoxic agent, pactamycin, has been isolated from *Streptomyces pactum* in this laboratory. Fermentation, isolation, and characterization are described in companion papers (Bhuyan, Smith, and Dietz, 1962; Argoudelis, Jahnke, and Fox, 1962).

Pactamycin has been chromatographed in a variety of solvents to establish a paper chromatographic pattern to use for subsequent identification. The pattern, established on Eagle's KB human epidermoid carcinoma cells in agar, was found to correlate with the pattern on *Bacillus subtilis* UC-564. A quantitative assay was developed using the latter organism.

Attempts were made to devise a quantitative spectroscopic assay similar to that used for other agents (Brown and Marsh, 1953), but the need for high doses of pactamycin precluded the use of an assay in the ultraviolet region. When it was discovered that the compound possessed a strong fluorescence, a paper chromatographic assay was designed to make use of this property.

A statistical comparison between the two assays was made to determine the reliability of the fluorescence method.

### Materials and Methods

Pactamycin was chromatographed on Whatman no. 1 paper in the seven solvent systems listed in Table 1. The strips, containing 0.35 $\mu$g of antibiotic per strip, were developed descending and, after drying for 1 hr, were plated on modified Miyamura agar trays seeded with $4 \times 10^5$ cells/ml of Eagle's KB carcinoma cells (Grady, Lummis, and Smith, 1960). The trays were incubated for 16 hr at 37 C with the strips in contact with the agar. On completion of incubation, the strips were removed and the agar sprayed with a 0.4% solution of 2,6-dichlorophenol-indophenol in methanol-saline (1:9; v/v). One hour was allowed for dye reduction, after which the trays were examined. The active zones appeared blue against a light blue or colorless background.

TABLE 1. *Paper chromatographic solvent systems*

| System | Composition |
|---|---|
| I | 1-Butanol-water (84:16; v/v) |
| II | 1-Butanol-water (84:16; v/v) plus 0.25% *p*-toluenesulfonic acid (w/v) |
| III | 1-Butanol-acetic acid-water (2:1:1; v/v) |
| VI | 1-Butanol-water (84:16; v/v) plus 0.2% (v) piperidine |
| V | 1-Butanol-water (4:96; v/v) |
| VI | 1-Butanol-water (4:96) plus 0.25% *p*-toluenesulfonic acid (w/v) |
| S-13 | *n*-Butyl acetate-1-butanol-10% aqueous formic acid (4:1:5) |

The biological assay was carried out on *B. subtilis* UC-564 in agar. One-half in. strips of Whatman no. 1 paper, containing 0.2, 0.4, 0.8, and 1.0 $\mu$g of pactamycin per strip, were developed descending in solvent, system S-13. The use of this solvent required equilibration of the strips for 16 hr before development. After development for 7 hr, the strips were dried, neutralized in an ammonia atmosphere, and plated on agar seeded with 0.3 ml of *B. subtilis* inoculum. The inoculum was prepared from *B. subtilis* spores and contained $3 \times 10^{10}$ cells per ml of suspension. Two unknown and three standard samples (purity greater than 95%) were plated on each tray and incubated for 16 hr at 30 C with the strips in contact with the agar. After removal of the strips, the width of the zones in the $R_F$ 0.7 region were measured and a standard curve constructed plotting the log dose per strip versus zone diameters of the standard samples.

The fluorescent assay required sample sizes of 1, 2, 4, and 6 $\mu$g of pactamycin per strip. The chromatographic technique for development of the strips was identical to that used for the biological assay. The fluorescent emission was recorded on a Cary spectrophotometer using a strip scanner (Parke and Davis, 1952). After drying, the $\frac{1}{2}$-in. Whatman no. 1 strips were scanned at 238 m$\mu$ with a 365-m$\mu$ sharp cut-off filter (Brown and Marsh, 1953) in the sample beam between the paper strip and the slit image focusing lens. A piece of Whatman no. 1 paper was used in the reference beam. The exciting wave length producing maximal fluorescent response was determined by scanning a chromatographed zone of pactamycin from 400 to 210 m$\mu$ with a 365-m$\mu$ filter in the sample beam and a sheet of paper in the reference beam. The density of paper used was just sufficient to produce a full-scale deflection on the spectrophotometer recorder with a 365-m$\mu$ filter in place and the balance potentiometer at mid-position. It was not necessary to use paper spotted with a fluorescent reference compound.

The area under the peaks at $R_F$ 0.7 was determined by the peak height times the half band width method. From these values and the log dose per strip, a standard curve was constructed.

## Results and Discussion

Figure 1 shows the paper chromatographic pattern of pactamycin on Eagle's KB human epidermoid carcinoma cells. An identical pattern was obtained on trays seeded with *B. subtilis*. Although KB cells were highly sensitive to pactamycin, the biological assay was developed on bacterial trays because *B. subtilis* was approximately ten times more sensitive to pactamycin than were KB cells. This eliminated the need for special treatment of trays.

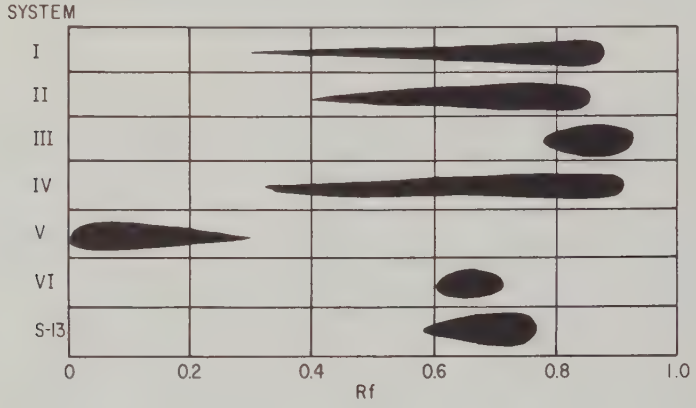


FIG. 1. *Pattern of pactamycin on KB cells in agar in various solvent systems.*

Brown and Marsh (1952) described the use of a filter in a sample beam of a spectrometer to detect zones of organic material on paper chromatograms. The exciting wave length used was 365 m$\mu$. Since this did not insure maximal fluorescent emission for all compounds, it was necessary to record an activation curve for pactamycin. Figure 2 is the activation curve, obtained as described in Materials and Methods. Standard curves for *B. subtilis* and the fluorescent assay are shown in Fig. 3 and 4. The percentage standard deviations for the curves are 4.0 and 6.8, respectively. The higher standard deviation for the fluorescent assay curve is probably due to variations in calculating areas under response peaks. The use of the spectrophotometer in the manner described results in a higher noise to signal ratio than usually encountered, and tracings are not as smooth as those usually obtained. With a little experience on the part of the operator, this does not prohibit a linear relationship between log dose response and area.

Based on very limited data, because of the lack of sufficient quantities of purified pactamycin, the accuracy of the assay methods did not differ by more than 5%. This was determined by chromatographing

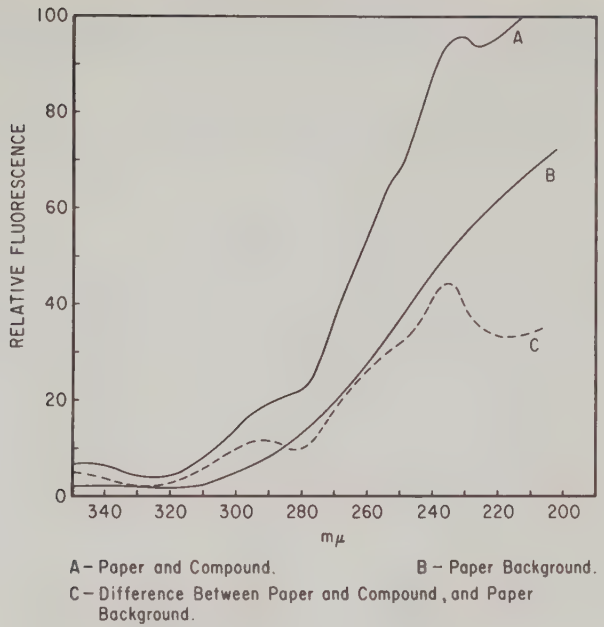


FIG. 2. *Activation curve for pactamycin.*

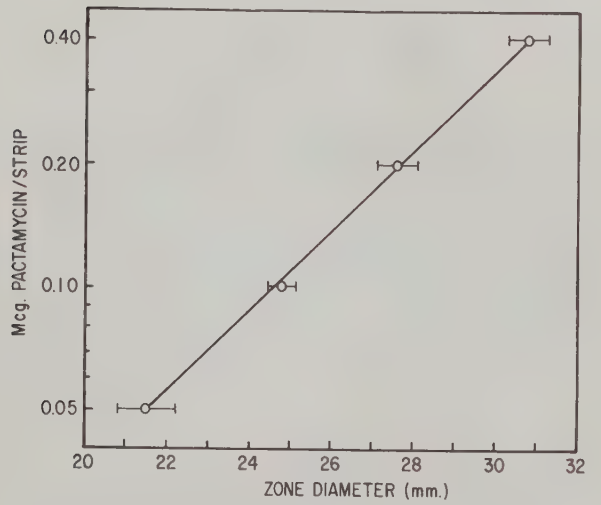


FIG. 3. *Pactamycin standard assay curve on Bacillus subtilis.*

known quantities of purified pactamycin mixed with other antibiotics and assaying the samples as described.

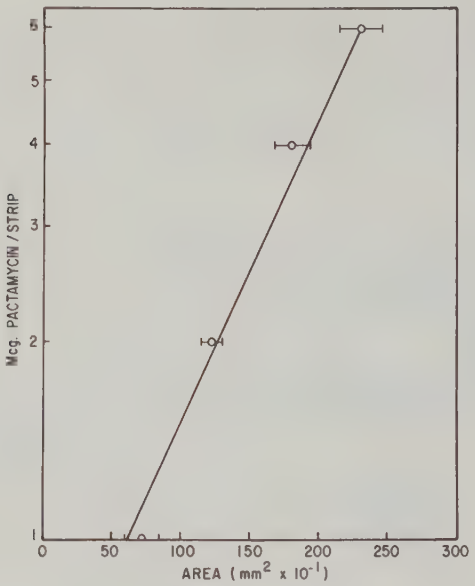


FIG. 4. *Pactamycin standard assay curve, determined by fluorescence.*

Table 2 shows the variation encountered between assay methods. Agreement is good considering the basic differences involved in interpreting results from each assay.

TABLE 2. *Variation between biological and fluorescent assays*

| Pactamycin, μg/mg | | Variation* 100 (b-a)/a |
|---|---|---|
| Fluor. (a) | Bio. (b) | |
| | | % |
| 1070 | 850 | -21 |
| 210 | 200 | - 5 |
| 205 | 190 | - 7 |
| 290 | 230 | -21 |
| 465 | 406 | -13 |
| 305 | 388 | +27 |
| 320 | 342 | + 7 |

*Mean: −5; standard deviation: 17.

Table 3 indicates the precision obtained with each assay. The 95% confidence limits were determined using a "t" distribution, $[\bar{x} + (t_{\frac{1}{2}\alpha}S)/N;\ \bar{x} + (t_{1-\frac{1}{2}\alpha}S)/N]$ (Dixon and Massey, 1957). The equality of the mean of each assay could not be tested by the pairing technique (Dixon and Massey, 1957), since the use of the same chromatograms in both assays was not possible. Using the formula

$$t = \frac{(\bar{x}_1 - \bar{x}_2) - (\mu_1 - \mu_2)}{(S_1^2/N_1) + (S_2^2/N_2)}$$

one obtains a value of t = 0.24 (Dixon and Massey, 1957). This value is then compared with a table giving percentiles for a "t" distribution. One rejects the hypothesis that $\mu_1$ is equal to $\mu_2$ if "t" is greater than +2.120 or less than -2.120 at a 5% level of significance.

TABLE 3. *Precision of fluorescent and bioassays**

| Fluorescent | Biological |
|---|---|
| 1000 | 857 |
| 792 | 775 |
| 968 | 1083 |
| 900 | 911 |
| 850 | 975 |
| 910 | 1051 |
| 963 | 771 |
| 954 | 989 |
| 923 | 1025 |

*Results are expressed as μg/mg of pactamycin. In the fluorescence assay, the mean was 931 and the standard deviation was 75. In the biological assay, the mean was 942 and the standard deviation was 115. The 95% confidence limits are ±53 μg/mg and ±81 μg/mg.

Although the data show a favorable comparison between the biological and fluorescent assay techniques for relatively high concentrations of pactamycin, the fluorescent assay cannot be used at this time for analysis of fermented culture media containing pactamycin. The levels of pactamycin in the maximal usable dose of culture media are generally below the quantities detectable in the fluorescent assay. It may be possible to concentrate culture filtrates prior to chromatography. The fluorescent assay proves of particular value in evaluating standards for assay curves, for it is possible to detect both biologically active and biologically inactive components in pactamycin preparations.

### Acknowledgment

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## Literature Cited

Argoudelis, A. D., H. K. Jahnke, and J. A. Fox. 1962. Pactamycin, a new antitumor antibiotic. II. Isolation and characterization. Antimicrobial Agents and Chemotherapy - 1961, p. 191-197.

Bhuyan, B. K., A. Dietz, C. G. Smith. 1962. Pactamycin, a new antitumor antibiotic. I. Discovery and biological properties. Antimicrobial Agents and Chemotherapy - 1961, p. 184-190.

Brown, J. A., and M. M. Marsh. 1953. Automatic measurement of light absorption and fluorescence on papergrams. Anal. Chem. **25**:1865-1868.

Brown, J. A., and M. M. Marsh. 1952. Paper chromatographic separation and determination of some water-soluble vitamins. Anal. Chem. **24**:1952-1953.

Dixon, W. J., and F. J. Massey, Jr. 1957. Introduction to statistical analysis, 2nd ed., McGraw-Hill Book Co., Inc., p. 123-128.

Grady, J. E., W. L. Lummis, and C. G. Smith. 1960. Tissue culture bioautographic system. Proc. Soc. Exptl. Biol. Med. **103**:727-730.

Parke, T. V., and W. W. Davis. 1952. Automatic spectrophotometry of paper strip chromatograms. Anal. Chem. **24**:2019-2020.

# EFFECT OF METAL CATIONS ON PRODUCTION OF THIOSTREPTON

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Thiostrepton is a weakly basic polypeptide antibiotic of low water solubility found in the mycelium of *Streptomyces azureus* (Pagano et al., 1956). It is highly active primarily against gram-positive bacteria. Recent studies have shown its molecular weight to be about 1,700. On acid hydrolysis, threonine, alanine, cysteine, thiostreptoic acid, and an unidentified chromophore have been obtained (Bodanszky et al., 1960). The isolated antibiotic is free of metallic elements.

In the development of media for thiostrepton fermentation, it was found that certain metal salts exerted profound stimulatory effects on antibiotic potencies. These effects are the subject of this paper.

## Materials and Methods

The *S. azureus* cultures were maintained as lyophilized spores in skim milk. Inocula were grown in a medium consisting of 5% extracted soybean flour, 0.8% cornsteep liquor, and 0.5% NaCl. The basic fermentation medium contained 4% extracted soybean flour, 7.5% glucose, 0.2% $CaCO_3$, and 0.5% $Na_2SO_4$. Shaken flask fermentations of 50 ml in 250-ml flasks were incubated at 25 C for 5 to 6 days. Fermentations of 30 liters were carried out under the same conditions in aerated stainless steel fermentors of 40-liter capacity (Kroll et al., 1956).

Cells of *S. azureus* for resting-cell experiments were harvested from the fermentation medium after 48 hr incubation. They were washed four times in 0.1% KCl and resuspended in the KCl solution at 45 mg/ml.

Antibiotic potencies were determined by a *Staphylococcus aureus* agar diffusion assay (Pagano et al., 1956) on dimethyl sulfoxide extracts of whole culture. Growth was estimated by determination of deoxyribonucleic acid (DNA) in whole broth (Burton, 1956). Glucose was determined by ferricyanide reduction (Hoffman, 1937) with the AutoAnalyzer (Technicon Instruments Corp., Chauncey, N.Y.). Total

keto acids were estimated colorimetrically (Friedemann and Haugen, 1943) and identified by paper chromatography of the 2,4-dinitrophenylhydrazones in butanol-1 saturated with 2 N $NH_4OH$. Iron, cobalt, and manganese were determined by quantitative spectrochemical assay.

## Results and Discussion

After a conventional study of carbohydrate and nitrogen sources for the fermentation, potencies of 100 to 200 mg/liter of thiostrepton could be obtained. Modifications of the major constituents of the basic fermentation medium failed to bring about further improvement in antibiotic production. When a study of trace-element requirements for the fermentation was made, it was quickly found that sulfates and chlorides of $Fe^{++}$, $Fe^{+++}$, $Co^{++}$, $Mn^{++}$, $Ni^{++}$, $Sn^{++}$, and $VO^{++}$, at levels of the magnitude of 0.001 M, stimulated thiostrepton production by 25 to 75% (Table 1). All other metal ions tested, $Ba^{++}$, $Cd^{++}$, $Ca^{++}$, $Mg^{++}$, $Cu^{++}$, $Zn^{++}$, $Al^{++}$, $Hg^{++}$, $Na^{+}$, $K^{+}$, $SeO_4^{--}$, $MoO_4^{--}$, $AsO_4^{--}$, and $Cr_2O_7^{--}$, were ineffective or inhibitory. The order of effectiveness of the active cations was $Fe^{++} > Co^{++} > Mn^{++} > VO^{++} > Ni^{++} > Sn^{++}$, although the effectiveness per millimole of cation added was $VO^{++} > Sn^{++} > Fe^{++} > Ni^{++} > Co^{++} > Mn^{++}$. Because of the inhibitory action of $VO^{++}$, $Sn^{++}$, and $Ni^{++}$, however, full advantage could not be taken of their greater intrinsic effects.

TABLE 1. *Effect of certain metal cations on the production of thiostrepton in shaken flasks*

| Cation | Optimal concn. | Potency* at optimal concn. |
|---|---|---|
| | *mM* | |
| $Fe^{++}$ | 1.1 | 340 |
| $Co^{++}$ | 1.1 | 330 |
| $Mn^{++}$ | 3.4 | 320 |
| $VO^{++}$ | 0.51 | 310 |
| $Ni^{++}$ | 0.40 | 280 |
| $Sn^{++}$ | 0.40 | 220 |
| Control | - | 170 |

*Expressed as mg/liter of thiostrepton.

$Co^{++}$, $Fe^{++}$, and $Mn^{++}$ salts were more effective when used in combination than when used singly, and, in fact, their effects were approximately additive when used at their optional concentrations (Table 2). Additive effects of $Sn^{++}$, $VO^{++}$, or $Ni^{++}$ could not be shown.

Ethylenediaminetetraacetic acid and relatively large amounts of phosphate obviated the effects of the effective cations. However,

TABLE 2. *Additive effects of $Fe^{++}$, $Co^{++}$, and $Mn^{++}$ at optimal concentrations on thiostrepton production in shaken flasks*

| Cation combinations | Calculated potency* | Actual potency† |
|---|---|---|
| $Co^{++}$, $Mn^{++}$ | 480 | 560 |
| $Co^{++}$, $Fe^{++}$ | 500 | 520 |
| $Fe^{++}$, $Mn^{++}$ | 490 | 510 |
| $Fe^{++}$, $Co^{++}$, $Mn^{++}$ | 650 | 610 |
| Control | - | 170 |

*Calculated potency = the sum of the potencies of each cation (Table 1) after the subtraction of the control (Table 1) from each value; the value of the control is then added to the total.

†Expressed as mg/liter of thiostrepton.

small amounts of phosphate enhanced the stimulation of a mixture of the effective, additive cations on potency. In addition, with 2 mM of added phosphate, 8 mM rather than 5.6 mM of total cation became the optimal concentration of added cations. It appeared that the cation-phosphate combination served as a noninhibitory reserve source of the cations. The optimal molar ratio of cations to phosphate was approximately 4 or 5 to 1 (Fig. 1).

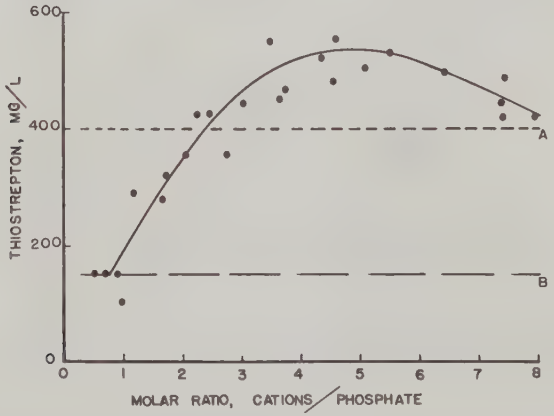


FIG. 1. *Effect of the ratio of added cations to added phosphate on thiostrepton production in shaken flasks. Controls were (A) 8.0 mM added cations, no phosphate, and (B) 2 mM added phosphate, no cations.*

Determinations of iron, cobalt, and manganese in fermentation broth demonstrated that little, if any, added cobalt or manganese was fixed by *S. azureus* cells (Table 3). Approximately 15% of the added iron, however, was found in the insoluble portion of the broth. It is

probable that the required levels of the cations reflected to a large degree the levels of soluble metal-complexing substances such as amino acids and peptides (Martin and Edsall, 1959) in the medium.

TABLE 3. *Distribution of added cations* in a 30-liter thiostrepton fermentation after 72 hr*

| Broth portion | Total recovered cation | | |
|---|---|---|---|
| | $Fe^{++}$ | $Co^{++}$ | $Mn^{++}$ |
| | % | % | % |
| Water soluble | 85 | 95 | 95 |
| Water insoluble | 15 | 5 | 5 |

*The cations, as sulfate salts, were added 24 hr after inoculation to provide the following concentrations: 1.1 mM $Fe^{++}$, 1.1 mM $Co^{++}$, and 3.4 mM $Mn^{++}$.

A suppression by the effective cations of the inhibitory effects of the antibiotic for *S. azureus*, such as observed with the tetracyclines and $Fe^{++}$ (Weinberg and Foley, 1955), is not consistent with the requirement for early addition of the cations because of the low concentration of antibiotic at this time. Further, thiostrepton added at the levels encountered in fermentations had no apparent effect on biosynthesis of the antibiotic, nor was there indication of degradation of the added antibiotic as determined by antibiotic assays.

TABLE 4. *Effects of $Fe^{++}$, $Co^{++}$, and $Mn^{++}$ on growth, glucose utilization, and keto acid formation by Streptomyces azureus in a 30-liter fermentation*

| Addition | Growth | Glucose utilization | Maximal keto acid level (α-ketoglutaric acid) |
|---|---|---|---|
| | *μg DNA/ml* | *mg/hr* | *μg/ml* |
| None (control) | 3.3 | 1.0 | 800 |
| Cations | 3.6 | 0.5 | 3,000 |

*The cations, as sulfate salts, were added 24 hr after inoculation to provide the following concentrations: 1.1 mM $Fe^{++}$, 1.1 mM $Co^{++}$, and 3.4 mM $Mn^{++}$.

In Table 4 are summarized some of the effects of a mixture of $Fe^{++}$, $Co^{++}$, and $Mn^{++}$ sulfate on thiostrepton fermentation. The growth rate was little affected, keto acids accumulated to a greater extent, and glucose utilization was retarded. Paper chromatography of the 2,4-dinitrophenylhydrazones of the keto acids showed them to be α-ketoglutaric and pyruvic acids in 4:1 ratio. Addition of either acid to the fermentation failed to affect the antibiotic potencies. Therefore, the increased accumulation of keto acids did not appear directly related to thiostrepton biosynthesis.

The stimulatory effect of the cations on the biosynthesis of thiostrepton was dependent on the time at which they were added to the fermentation (Table 5). Additions of the cations made later than 24 hr after inoculation were practically without effect on potency. Resting cells which had been grown in the presence of the effective cations were always capable of greater rates of antiobiotic formation than were cells grown without added cations (Fig. 2). Addition of the

TABLE 5. *Effect of time of addition of a mixture of $Fe^{++}$, $Co^{++}$, and $Mn^{++}$ on thiostrepton thiostrepton production in shaken flasks*

| Time of cation* addition | Fermentation potency |
|---|---|
| *hr* | |
| Inoculation | 750 |
| 24 | 900 |
| 48 | 340 |
| 72 | 190 |
| 96 | 180 |
| No salts added | 140 |

*The cation mixture provided 1.1 mM $FeSO_4 \cdot 7H_2O$, 1.1 mM $CoSO_4 \cdot 7H_2O$, and 3.4 mM $MnSO_4 \cdot H_2O$.

†Expressed as mg/liter of thiostrepton.

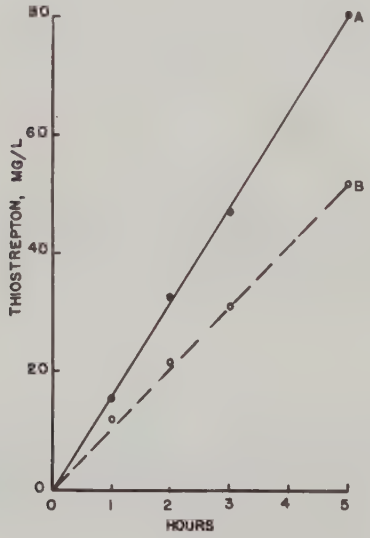


FIG. 2. *Thiostrepton formation by washed cells of Streptomyces azureus which were grown (A) in the presence of added $Fe^{++}$, $Co^{++}$, and $Mn^{++}$, and (B) in the absence of added cations.*

cations to the latter cells did not affect their rates of antibiotic formation. It is evident that the cations do not act as cofactors of pre-existing enzyme systems for the biosynthesis of thiostrepton. These results are similar to those of Shankar and Bard (1955), which indicated that *Clostridium perfringens* grown in the presence of cobalt synthesized a homo-lactic pathway for glucose dissimilation. This massive diversion of enzyme pathways was attributed to altered enzyme synthesis or induction. This phenomenon is also consistent with a rapid genetic alteration of the culture as a result of a rapidly occurring selective effect. Although growth of *S. azureus* was not affected by the cations, three transfers of inocula in cation-supplemented medium resulted in cultures able to produce greater antibiotic potencies than cultures transferred in the same manner in unsupplemented medium (Table 6). The data thus indicate that the pattern of enzyme synthesis is *S. azureus* was altered by growth in the presence of the cations and that selective effects probably contributed to the expression of this mechanism.

TABLE 6. *Effect of inoculum growth conditions on thiostrepton production in shaken flasks*

| Inoculum grown for 3 passages in | Fermentation Potency* |
|---|---|
| Standard inoculum medium | 520 |
| Cation inoculum medium* | 980 |

*The fermentation and cation inoculum media were supplemented with 1.1 mM $FeSO_4 \cdot 7H_2O$, 1.1 mM $CoSO_4 \cdot 7H_2O$, and 3.4 mM $MnSO_4 \cdot H_2O$.

## Acknowledgments

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## Literature Cited

Bodanszky, M., J. J. Sheehan, J. Fried, N. J. Williams, and C. A. Birkhimer. 1960. Degradation of thiostrepton. Thiostreptoic acid. J. Am. Chem. Soc. **82**:4747-4748.

Burton, K. 1956. A study of the conditions and mechanism of the diphenylamine reaction for the colorimetric estimation of deoxyribonucleic acid. Biochem. J. **62**:315-323.

Friedemann, T. E., and G. E. Haugen. 1943. Pyruvic acid. II. The determination of keto acids in blood and urine. J. Biol. Chem. **147**:415-441.

Hoffman, W. S. 1937. A rapid photoelectric method for the determination of glucose in blood and urine. J. Biol. Chem. **120**:51-55.

Kroll, C. L., S. Formanek, A. S. Covert, L. A. Cutter, J. M. West, and W. E. Brown. 1956. Equipment for small scale fermentations. Ind. Eng. Chem. **48**:2190-2193.

Martin, R. B., and J. T. Edsall. 1959. Association of bivalent cations with glutathione. J. Am. Chem. Soc. 81:4044-4047.

Pagano, J. F., M. J. Weinstein, H. A. Stout, and R. Donovick. 1956. Thiostrepton, a new antibiotic. I. In vitro studies. Antibiotics Ann. 1955-56, p.554-559.

Shankar, K., and R. C. Bard. 1955. Effect of metallic ions on the growth, morphology, and metabolism of *Clostridium perfringens*. II. Cobalt. J. Bacteriol. 69:444-448.

Weinberg, E. D., and M. S. Foley. 1955. The effect of metallic cations on the anti-streptomyces activity of anhydrochlortetracycline. J. Pharmacol. Exptl. Therap. 115:61-67.

# EFFECT OF MEDIUM COMPOSITION ON AMPHOTERICIN B PRODUCTION BY *STREPTOMYCES NODOSUS*

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The two polyene antifungal antibiotics, amphotericins A and B, are produced by *Streptomyces nodosus* (Gold et al., 1956; Vandeputte, Wachtel, and Stiller, 1956). These antibiotics were shown to be useful therapeutic agents in deep-seated and superficial mycoses (Steinberg, Jambor, and Suydam, 1956). Amphotericin B, the antibiotic of choice, was found to be difficult to isolate in the presence of high concentrations of amphotericin A. It thus became necessary to devise a fermentation process in which high amphotericin B and low amphotericin A titers could be achieved. This paper will describe some of the biochemical methods employed to control the particular amphotericins produced.

## Materials and Methods

*S. nodosus* was maintained by weekly transfer on Carvajal's agar (Hesseltine, Benedict, and Pridham, 1954) with added asparagine (0.1%). Permanent stocks were made by lyophilizing spores grown on this agar in 0.1% sterile lecithin solution. Vegetative inoculum was produced by inoculating with a loopful or vial of spores a 250-ml Erlenmeyer flask containing 50 ml of medium consisting of (in g per liter): Staley's 4S nutrient (A. E. Staley Manufacturing Co., Decatur, Ill.) 30; glucose, 50; and $CaCO_3$, 7. After 3 days of incubation at 25 C on a shaker (B. F. Gump Co., Chicago, Ill.), rotating at 280 rev/min with a 2-in. stroke, 10% of the contents of these flasks were transferred to a second series of flasks containing the same medium. Fermentation flasks were inoculated from this second vegetative stage after a 2-day growth period. All flask fermentations were carried out in 250-ml Erlenmeyer flasks containing 50 ml of medium at 25 C on a Gump shaker. Inocula for 30-liter and 3,024-liter fermentations were prepared in a similar manner, with the addition of a third laboratory stage consisting of 1 liter of medium in a 4-liter Erlenmeyer flask inoculated with 100 ml of second vegetative

stage material. After 2 days of incubation at 25 C on a reciprocating shaker (Day Co., Cincinnati, Ohio) (1.5- to 2.0-in. stroke at 120 strokes per min), the third inoculum stage was used to inoculate 30-liter fermentations directly, or to inoculate 226 liters of a similar broth which, in turn, could be used to inoculate several fermentors after a 2-day growth period. Medium for the tank-scale inocula contained (in g per liter): extracted soybean meal, 20; glucose, 50; $CaCO_3$, 5; and a polyalkylene glycol, Ucon Lubricant LB625 (Union Carbide Corp., New York, N.Y.), 0.5.

Agitator power input in the fermentors was 0.05 metric horsepower per 100 liters, and the air flow was 0.5 m per min superficial air velocity in the inoculum stage of fermentation. The temperature was maintained at 30 C. Foam was controlled by the addition of lard oil.

Fermentations in 30-liter tanks were carried out in the equipment described by Kroll et al. (1956). The tank inoculum stage and the 3,024-liter fermentation were carried out in larger equipment of similar design.

The antibiotics produced were measured by the assay procedures of Gold et al. (1956).

## Results and Discussion

*Effect of carbohydrate on amphotericin produced.* The data indicate that, with 1% glucose, more amphotericin B than A is produced (Table 1). As the amount of glucose in the medium is increased, the yield of amphotericin A production is increased. It is obvious that direct application of these results to control the type of amphotericin produced (particularly amphotericin B) would be impractical because of the lower total yield of antibiotic produced when the amount of carbohydrate is reduced.

TABLE 1. *Effect of the amount of glucose in the medium* on the type of amphotericin produced*

| Glucose | Amphotericins at 120 hr† | | Ratio of amphotericin B to A |
|---|---|---|---|
| | A | B | |
| % | *μg/ml* | *μg/ml* | |
| 1 | 510 | 690 | 1.35 |
| 2 | 970 | 900 | 0.92 |
| 3 | 1,690 | 1,125 | 0.66 |
| 4 | 2,475 | 1,260 | 0.50 |
| 5 | 3,025 | 1,420 | 0.46 |

*Extracted soybean meal (30 g per liter) and $CaCO_3$ (7 g per liter).

†An average chemical assay of two fermentation flasks.

Carbohydrate utilization by microorganisms can be controlled if the carbohydrate is added intermittently or continuously throughout the fermentation period. Such a method has been used for the production of penicillin (Soltero and Johnson, 1953). Table 2 summarizes the results of a study on intermittent-glucose addition to shaken-flask fermentations. This technique resulted in only slightly increased titers of amphotericin B, but markedly reduced levels of amphotericin A produced.

TABLE 2. *Effect of intermittent carbohydrate addition on amphotericin production in shaken flasks**

| Initial glucose | Glucose added per 24 hr, starting at 24 hr | Amphotericin at 120 hr† | | Ratio amphotericin B to A |
|---|---|---|---|---|
| | | A | B | |
| % | % | *μg/ml* | *μg/ml* | |
| 5 | 0 | 8,000 | 3,000 | 0.37 |
| 1 | 1 | 4,600 | 3,250 | 0.70 |
| 1 | 2 | 6,200 | 4,000 | 0.64 |
| 2 | 1 | 5,100 | 4,100 | 0.80 |

*Flasks contained 20 g Pharmamedia (Trader's Protein Division, Trader's Oil Mill Co., Fort Worth, Texas) and 7 g $CaCO_3$ per liter.
†An average chemical assay of two fermentation flasks.

Another method of regulating carbohydrate metabolism involves the use of a slowly utilized carbohydrate. An example of this approach is the use of lactose in penicillin-producing fermentations (Jarvis and Johnson, 1947). Various carbohydrates were tested in the amphotericin fermentation, and a dextrin known as British Gum was selected as giving the most satisfactory results. Combinations of glucose and British Gum were found to give the highest yields of amphotericin in 30-liter fermentations (Table 3). In addition to high yields of amphotericin B, a ratio favoring amphotericin B was obtained with this carbohydrate.

TABLE 3. *Amphotericin production in 30-liter fermentations with British Gum as a carbohydrate source**

| British Gum | Glucose | Amphotericin at 120 hr | | Ratio of amphotericin B to A |
|---|---|---|---|---|
| | | A | B | |
| % | % | *μg/ml* | *μg/ml* | |
| 0 | 5.0 | 4,050 | 1,100 | 0.27 |
| 8 | 0.0 | 414 | 1,180 | 2.85 |
| 8 | 1.1 | 840 | 2,530 | 3.01 |
| 6 | 0.55 | 334 | 1,220 | 3.65 |
| 4 | 0.0 | 213 | 930 | 4.36 |
| 4 | 1.1 | 482 | 1,560 | 3.23 |

*Medium contained 35 g Pharmamedia and 7 g $CaCO_3$ per liter.

*Effect of fats, fatty acids, and alcohols.* Certain fats and fatty acids can be utilized as energy sources in the production of the polyene, filipin (Brock, 1956). A large number of fats, fatty acids, and higher alcohols were tested alone, or with glucose, as energy sources in the amphotericin fermentation. Among those tested were sodium oleate, methyl oleate, glycerol oleate, lard oil, corn oil, peanut oil, octadecanol, polyoxyethylene sorbitan monooleate (Atlas Powder Co., Wilmington, Del.), and glycerol monooleate. None of these materials gave better antibiotic yields than glucose alone.

*Effect of inorganic constituents.* The effects of the inorganic constituents of the medium were also investigated. Table 4 summarizes the results obtained in shaken flasks when the amount of $CaCO_3$ was varied. Omission of $CaCO_3$ reduced the amount of amphotericin A and increased the viscosity of the fermentation broth. This effect on antibiotic production was also observed in 30-liter fermentations when glucose was used as the carbohydrate source. In fermentations without $CaCO_3$, the pH fell to 5.8; control fermentations remained at pH 6.5 or above.

TABLE 4. *Effects of calcium carbonate level on amphotericin production in shaken flasks**

| Calcium carbonate | Viscosity, centipoises | Amphotericin at 120 hr | | Ratio of amphotericin B to A |
|---|---|---|---|---|
| | | A | B | |
| % | | *μg/ml* | *μg/ml* | |
| 0.0 | 307 | 2,016 | 1,770 | 0.87 |
| 0.1 | 182 | 4,950 | 1,940 | 0.39 |
| 0.2 | 167 | 5,170 | 2,050 | 0.39 |
| 0.5 | 170 | 5,450 | 2,160 | 0.39 |
| 1.0 | - | 5,230 | 2,160 | 0.41 |
| 2.0 | 167 | 5,180 | 2,530 | 0.48 |

*Flasks contained extracted soybean meal (Archer-Daniels-Midland, Minneapolis, Minn.), 30 g per liter, and glucose, 50 g per liter.

Magnesium, calcium, and iron salts were found to have little effect on antibiotic titers. Nickel, zinc, and cobalt salts increased the titers of both amphotericin A and B. Manganous salts, especially in presence of iron salts, however, were the most effective in causing this stimulation (Table 5). However, it was subsequently found that isolation of highly purified amphotericin was difficult when the fermentation medium contained 0.03% each of manganous sulfate and ferrous sulfate, and British Gum was the primary carbohydrate source.

*Pilot plant production.* As mentioned earlier, it was found necessary to control the amounts of amphotericins A and B in the fermentation, because of the difficulty of separating mixtures of the two antibiotics. This control can be exerted by the selection of the

TABLE 5. *Effect of iron and manganous salts on amphotericin production in a Britsh Gum-containing medium**

| Method | Salts | | Amphotericin | | Ratio of amphotericin B to A |
|---|---|---|---|---|---|
| | $MnCl_2 \cdot 4H_2O$ | $FeSO_4 \cdot 7H_2O$ | A | B | |
| | % | % | *μg/ml* | *μg/ml* | |
| Shaken flasks | 0.0 | 0.0 | 1,180 | 1,050 | 0.88 |
| | 0.008 | 0.0 | 1,400 | 1,540 | 1.10 |
| | 0.0 | 0.008 | 1,600 | 1,120 | 0.70 |
| | 0.008 | 0.008 | 1,430 | 1,880 | 1.31 |
| | 0.032 | 0.032 | 1,260 | 2,100 | 1.66 |
| 30-Liter fermentations | | | | | |
| | 0.0 | 0.0 | 1,080 | 1,460 | 1.35 |
| | 0.02 | 0.02 | - | 1,950 | - |
| | 0.04 | 0.04 | 740 | 2,080 | 2.81 |

*Extracted soybean meal, 30 g; $CaCO_3$, 7 g; and British Gum, 50 g per liter.

amount and type of carbohydrate used. For example, with 7% glucose, the production of amphotericin A is favored, and with 5% British Gum, a broth suitable for the isolation of amphotericin B is obtained. A product containing approximately equal portions of amphotericins A and B can be isolated from fermentations in which 2.5% British Gum and 2.5% glucose are used. This scheme of biochemical control was used for the production of amphotericin in the pilot plant in 3,024-liter fermentations (Table 6).

TABLE 6. *Production of amphotericin for the isolation of amphotericin A and B mixtures in 3,024-liter fermentations**

| Amphotericin | | Ratio of amphotericin B to A |
|---|---|---|
| A | B | |
| *μg/ml* | *μg/ml* | |
| 1,860 | 2,320 | 1.24 |
| 2,460 | 1,670 | 0.67 |
| 2,280 | 1,990 | 0.87 |
| 2,230 | 1,920 | 0.86 |
| 1,560 | 1,400 | 0.89 |
| 2,900 | 1,860 | 0.64 |
| 2,570 | 2,040 | 0.79 |
| 2,266 (Avg) | 1,885 (Avg) | 0.851 (Avg) |

*Medium contained: British Gum, 25 g: glucose, 25 g; Pharmamedia, 35 g; and $CaCO_3$, 7 g per liter.

Isolation of a product containing a greater amount of amphotericin B can be obtained more readily when the fermentation broth contains considerably more amphotericin B than A. For this purpose, a medium containing 5% British Gum was used in the pilot

plant in 3,024-liter fermentations (Table 7). In general, the fermentations were controlled to produce the desired ratio of amphotericin B.

TABLE 7. *Production of amphotericin for the isolation of amphotericin B in 3,024-liter fermentation**

| Amphotericin | | Ratio of amphotericin B to A |
|---|---|---|
| A | B | |
| *μg/ml* | *μg/ml* | |
| 1,630 | 1,146 | 0.70 |
| 770 | 828 | 1.07 |
| 1,150 | 1,870 | 1.62 |
| 1,460 | 1,700 | 1.16 |
| 1,270 | 1,360 | 1.07 |
| 1,560 | 1,350 | 0.86 |
| 775 | 2,040 | 2.63 |
| 1,080 | 1,840 | 1.70 |
| 550 | 1,975 | 3.59 |
| 1,138 (Avg) | 1,568 (Avg) | 1.38 (Avg) |

*Medium contained: British gum, 50 g; glucose, 4 g; Pharmamedia, 35 g; and $CaCO_3$, 7 g per liter.

This limited experience at the pilot plant scale demonstrates the degree of control which can be exerted over the process by regulating the carbohydrate.

Although this approach enabled us to achieve our objective, it is our belief that, ultimately, better means of controlling the fermentation must be found.

## Literature Cited

Brock, T. D. 1956. The effect of oils and fatty acids on the production of filipin. Appl. Microbiol. 4:131-133.

Gold, W., H. A. Stout, J. F. Pagano, and R. Donovick. Amphotericins A and B, antifungal antibiotics produced by a streptomycete. I. In vitro studies. Antibiotics Ann. 1955-1956, p. 579-586.

Hesseltine, C. W., R. G. Benedict, and T. G. Pridham. 1954. Useful criteria for species differentiation in the genus *Streptomyces*. Ann. N. Y. Acad. Sci. 60:136-151.

Jarvis, F. G., and M. J. Johnson. 1947. The role of the constituents of synthetic media for penicillin production. J. Am. Chem. Soc. 69:3010-3017.

Kroll, C. L., S. Formanek, A. S. Covert, L. A. Cutter, J. M. West, and W. E. Brown. 1956. Equipment for small scale fermentations. Ind. Eng. Chem. 48:2190-2193.

Soltero, F. V., and M. J. Johnson. 1953. The effect of carbohydrate nutrition on penicillin production by *Penicillium chrysogenum* Q-176. Appl. Microbiol. 1:52-56.

Steinberg, B. A., W. P. Jambor, and L. O. Suydam. Amphotericins A and B; two new antifungal antibiotics possessing high activity against deep-seated and superficial mycoses. Antibiotics Ann. 1955-1956, p. 574-578.

Vandeputte, J., J. L. Wachtel, and E. T. Stiller. Amphotericins A and B, antifungal antibiotics produced by a streptomycete. II. The isolation and properties of the crystalline amphotericins. Antibiotics Ann. 1955-1956, p. 587-591.

# MODE OF ACTION OF VANCOMYCIN ON *STAPHYLOCOCCUS AUREUS*

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Vancomycin, an antibiotic produced by *Streptomyces orientalis*, is effective against a number of growing gram-positive bacteria and some spirochetes. It is bactericidal, and the development of resistance towards it, especially by *Staphylococcus aureus*, is considerably less frequent than that observed with many other antibiotics (Garrod and Waterworth, 1956; Ziegler, Wolfe, and McGuire, 1956). Since it is clinically effective against strains of *S. aureus* and resistant to the more common antibiotics (Kirby and Divelbiss, 1959), studies on its mode of action are of some interest.

In a preliminary report, Jordan and Inniss (1959) stated that vancomycin caused a decided inhibition of ribonucleic acid (RNA) production in growing cells of a pathogenic staphylococcus, whereas the synthesis of deoxyribonucleic acid (DNA) and protein remained relatively undisturbed for a well-defined period of time. The present investigation was prompted by our observation that, although this antibiotic exerted no early effect on the synthesis of protein as determined by the Folin-Ciocalteu method, it did inhibit the incorporation, by growing cells, of $C^{14}$-glutamic acid into a "bound" form which remained in the cells after the intracellular pool materials and the nucleic acid had been extracted. This suggested that the antibiotic interfered with the synthesis of a polypeptide devoid of aromatic amino acids, and directed attention to the cell-wall mucopeptide.

## Materials and Methods

*Media and drugs.* Vancomycin (free base) was obtained through the courtesy of the Eli Lilly Company, Indianapolis, Ind. The synthetic medium used in much of this work was composed of: glucose, 5 g; vitamin-free Casamino acids, 2 g; DL-tryptophan, 20 mg; thiamine hydrochloride, 2 mg; nicotinamide, 2 mg; $K_2HPO_4$, 0.5 g; $MgSO_4 \cdot 7H_2O$, 0.2 g; $(NH_4)_2SO_4$, 0.5 g; and glass-distilled water, 1000 ml. The pH was adjusted to 7.0 with 1.5 N NaOH. In one

modification, the tryptophan level was reduced to 0.5 gm/liter; in another, the phosphate level was reduced to 0.1 g/liter. All incubations were carried out at 37 C.

*Resistance studies.* In an effort to obtain vancomycin-resistant strains of *S. aureus* for comparison with sensitive strains, the antibiotic spectra of 285 clinical isolates obtained from various Canadian and American institutions were determined by the disc method. In addition, a strain of staphylococcus resistant to penicillin, dihydrostreptomycin, and the tetracyclines was subjected to a dose of X rays sufficient to cause a 99.97% kill, and cultures obtained from 265 survivors were tested by both disc and tube methods.

*Growth studies.* Brain heart infusion (Difco), inoculated with *S. aureus* 71435 (the strain used in all the following work), was incubated for 4 hr, at which time vancomycin was added. A control culture contained no vancomycin. Protoplasmic growth was measured by a Coleman photonephelometer; viable and total cell counts were made by standard plating procedures and by the use of a Petroff-Hauser chamber, respectively.

*Cellular leakage and the "nucleotide" pool.* The cells in 150 ml of a 15-hr culture in a low-tryptophan medium were removed by centrifugation and suspended in 10 ml of clear supernatant fluid. The resulting suspension was divided into three 3-ml portions, and the final volumes raised to a total of 9 ml by the addition of fresh medium. Vancomycin was added to one portion. One of the samples without antibiotic was immediately Millipore-filtered; the remaining samples were incubated 2 hr prior to filtration. The concentration of vancomycin remaining in the one filtrate was determined by a spectrophotometric procedure based on the strong ultraviolet absorption of vancomycin in the wave length region of 281 m$\mu$, and a suitable spectrophotometric "blank," consisting of an equivalent amount of antibiotic in water was prepared. Spectral-transmittance curves were determined for the three filtrates and for the synthetic medium.

For analysis of the "nucleotide" pool, the cells harvested from 4 liters of synthetic medium were suspended in 17 ml of original supernatant fluid, and 8.5 ml of this suspension were added to each of two 600-ml amounts of synthetic medium, one of which contained 85 $\mu$g vancomycin/mg cell dry wt. At various time intervals, 150-ml samples were removed, immediately frozen in a dry ice-alcohol mixture, and, after rapid thawing, centrifuged at 5 C; the harvested cells were washed twice in ice-cold water. The pool constituents were extracted from the cells by 0.25 M perchloric acid, treated with Norit A (Tsuboi and Price, 1959), and the adsorbed materials were eventually eluted with 10% aqueous pyridine and evaporated to dryness in a stream of cool air. The residue was then taken up in a fixed volume of 10% pyridine and subjected to chromatographic analysis (Dorough and Seaton, 1954). The separated compounds were located on the paper by their ultraviolet absorption; they were then eluted with 0.1 N HCl and spectrophotometrically examined.

*Synthesis of nucleic acid, protein, and cell-wall mucopeptide.* All radioactivity measurements were made in a flow-counter provided with an ultrathin plastic window. The $C^{14}$ was counted at infinite thinness.

A 10-ml 18-hr brain heart infusion culture of *S. aureus* was added to 90 ml of low-phosphate synthetic medium and incubated for 24 hr. The cells were removed by centrifugation, suspended in 1.8 ml of water, and 0.5-ml amounts were added to two flasks, each containing 27.5 ml of low-phosphate medium and 1 ml (1.38 $\mu$c) of inorganic $P^{32}$. After incubation under continuous agitation for 30 min, 1.0 ml of aqueous vancomycin solution was added to one flask and an equal volume of water to the other. Periodically, 5-ml samples were removed and immediately frozen. After rapid thawing, the cells were harvested, washed, and fractionated by the method of Hanawalt (1959), modified to include an ethanol-ether (3:1) extraction, and the total nucleic acid-$P^{32}$ and the DNA-$P^{32}$ measured.

For studies on the rate of synthesis of protein, wall mucopeptide, and several other cellular fractions, the incubation mixture was the same as that indicated for the nucleic acid analyses, except that ordinary synthetic medium replaced the low-phosphate medium and a $C^{14}$-amino acid (0.5 $\mu$c glycine-2-$C^{14}$, 3.5 $\mu$c DL-glutamic acid-2-$C^{14}$, or 3.5 $\mu$c DL-glutamic acid-3,4-$C^{14}$) replaced the $P^{32}$. After a 10-min incubation period, vancomycin was added to one sample, and, at various time intervals, 5-ml samples were removed, frozen, and eventually fractionated by a slight modification of the method of Park and Hancock (1960).

## Results

Although the number of clinical isolates resistant to dihydrostreptomycin and penicillin was large (31 and 20%, respectively), none of these isolates was resistant to 2 $\mu$g of vancomycin (supplied on a sensitivity disc) and none of the X-ray survivors was stably resistant to more than 5 $\mu$g/ml. Attempts to isolate resistant mutants through the use of the gradient-plate technique were unsuccessful.

Within 30 min of the addition of the antibiotic to logarithmic-phase cells, the total cell count became constant and the viable count rapidly decreased, whereas the culture nephelos continued to increase for a period of about 60 min (Fig. 1). These results suggested that during the initial stages of its activity vancomycin prevented cellular division without a concurrent blockage of protoplasmic synthesis.

During the growth of *S. aureus* in synthetic medium, there was a leakage of 260 m$\mu$-absorbing material from the cells (Fig. 2).

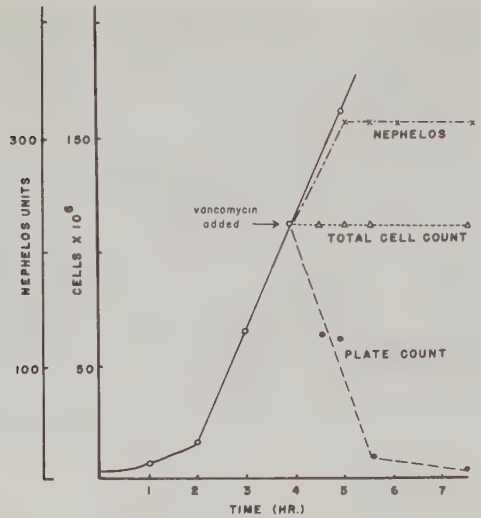


FIG. 1. *Effect of vancomycin, at a concentration of 10 μg/ml, on logarithmic-phase cells of Staphylococcus aureus. The control curve (without vancomycin present) is recorded here in nephelos units, but is paralleled by curves representing the total cell count and plate count.*

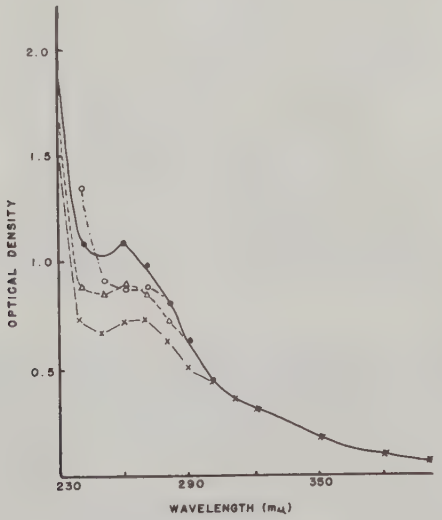


FIG. 2. *Spectral-transmittance of broth supernatants after incubation with Staphylococcus aureus in the presence or absence of vancomycin.* × = *uninoculated synthetic medium (blank = water);* Δ = *supernatant, no vancomycin (blank = water);* ● = *supernatant, no vancomycin, incubated 2 hr (blank = water);* ○ = *supernatant, plus 100 μg vancomycin/ml, incubated 2 hr (blank = water containing 28 μg vancomycin/ml).*

This loss was growth-dependent and did not occur in nongrowing cells suspended in water. The addition of vancomycin reduced the leakage, possibly by limiting the number of cells present (Eaton and Caffrey, 1961), and no gross increase in cellular permeability was observed.

During 30 min of normal growth, the majority of the seven ultraviolet-absorbing compounds found in the intracellular pool increased in concentration, so that at this stage the rate of synthesis of these compounds apparently was greater than their rate of removal from the pool. Vancomycin caused no qualitative differences in these pool constituents, but it augmented the concentrations of at least six of the components between 10 and 30 min after its addition. During the same time, the antibiotic rapidly decreased the rate of $P^{32}$ incorporation into RNA; a smaller decrease in the incorporation into DNA was observed a short time later (Fig. 3).

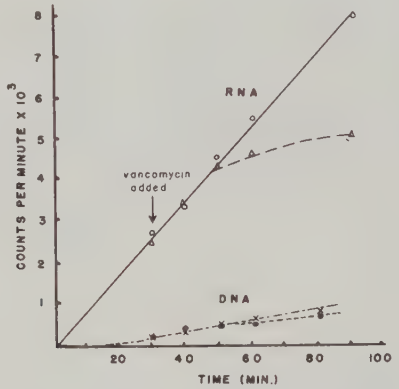


FIG. 3. *Effect of vancomycin at a concentration of 83 μg/mg cell dry wt on the incorporation of inorganic $P^{32}$ into the nucleic acids of Staphylococcus aureus.* ○ = *RNA, no vancomycin;* △ = *RNA, plus vancomycin;* × = *DNA, no vancomycin;* ● = *DNA, plus vancomycin.*

Vancomycin had no marked effect on the incorporation of $C^{14}$-amino acids into the "ethanol-soluble" protein and lipid of *S. aureus* (fraction 2 of Park and Hancock, 1960) or on the passage of these amino acids into the intracellular pool (fraction 1). This latter aspect was confirmed by growing cells for various time intervals in media containing $C^{14}$-glycine or $C^{14}$-DL-glutamic acid in the presence and absence of vancomycin and determining the $C^{14}$ content of the pool constituents extracted from these cells by the hot-water method of Cohen and Rickenberg (1956). Similar studies revealed that the antibiotic did not alter the uptake of $Co^{60}$, $Fe^{59}$, or $P^{32}$.

The lack of any effect of vancomycin on the incorporation of glycine-2-$C^{14}$ into trypsin-solubilized protein, and its striking inhibition of the incorporation of this amino acid into the wall mucopeptide, are illustrated in Fig. 4. Similar results were obtained with $C^{14}$-DL-glutamic acid except that the rate of uptake into the mucopeptide was slower, which in these steady-state, logarithmic-phase cultures probably reflects the known fact that staphylococcal cell walls contain more glycine than glutamic acid. An inhibition of mucopeptide synthesis occurred at a vancomycin level as low as 17 $\mu$g/ml cell dry wt, and was absolute within 2 to 4 min at a level of 83 $\mu$g/ml cell dry wt. No ameliorative effect on this inhibition was observed when the following compounds were added to the test system just prior to the vancomycin: uridine, thymidine, cytidine, adenosine, guanosine, uridine di- and triphosphate, adenosine mono-, di, and triphosphate, di- and triphosphopyridine nucleotides, coenzyme A, $\alpha$-lipoic acid, orotic acid, ureidosuccinic acid, ribose, deoxyribose, *N*-acetylglucosamine, sodium lactate, D-alanine, 1 M NaCl, DNA, RNA, and RNA hydrolyzed for 4 to 16 hr in 0.3% KOH and neutralized.

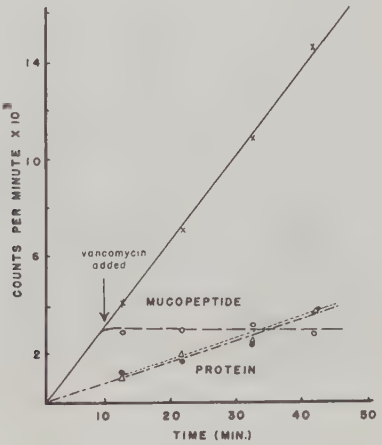


FIG. 4. *Effect of vancomycin at a concentration of 83 $\mu$g/mg cell dry wt on the incorporation of glycine-2-$C^{14}$ into the cell-wall mucopeptide and trypsin-solubilized protein of Staphylococcus aureus.* × = *mucopeptide, no vancomycin;* ○ = *mucopeptide, plus vancomycin;* ● = *protein, no vancomycin;* Δ = *protein, plus vancomycin.*

## Discussion

The data presented suggest that the primary effect of vancomycin on *S. aureus* is the inhibition of the production of cell-wall mucopeptide.

Death could occur solely by reason of the resulting malfunctioning division process, although the inhibition of RNA synthesis which occurs at a later stage is probably a contributing factor. This prevention of RNA formation appears to be a secondary effect of vancomycin action, brought about by the initial blockage of mucopeptide production. It has been proposed (Park and Strominger, 1957) that a nucleic acid precursor, uridine diphosphate, functions as a carrier of *N*-acetylmuramic acid peptide, a precursor of the mucopeptide component of the walls of gram-positive bacteria. Consequently, if the production of uridine diphosphate was prevented, the mechanisms for both cell-wall and RNA synthesis would fail. However, this hypothesis is questionable relative to vancomycin activity, because the presence of uridine diphosphate in an amount greater than that of the antibiotic failed to prevent the inhibition of either of these two anabolic processes.

The apparent lack of any large-scale permeability changes indicates that death by vancomycin is not a result of cellular lysis, a fact also borne out by the constancy of the total cell counts for 3.5 hr after vancomycin addition.

The observed impairment of DNA synthesis is probably only distantly related to the primary lesion caused by vancomycin, since it did not occur until the cells had been in contact with the antibiotic for about 30 min, at which point the cells were rapidly dying.

Superficially, the activity of vancomycin is similar to that of penicillin, in that there is an interference in the synthesis of both cell wall and RNA without a concomitant effect on protein production. Penicillin, however, in the absence of a stabilizing agent such as 1 M NaCl (Hancock, 1958), prevents the uptake of glutamic acid by staphylococci (Gale and Taylor, 1947), whereas vancomycin does not.

Since the action of vancomycin on mucopeptide synthesis is not prevented by the presence of increased amounts of the more usual metabolic intermediates, information on the exact enzyme system inhibited must await elucidation of the chemical nature of accumulated precursors and future developments in the field of cell-wall biosynthesis.

## Acknowledgments

The financial help of the National Research Council of Canada and the technical assistance of C. Mallory are gratefully acknowledged.

## Literature Cited

Cohen, G. N., and H. V. Rickenberg. 1956. Concentration spécifique réversible des amino acides chez *Escherichia coli,* Ann. inst. Pasteur **91**:693-720.

Dorough, G. D., and D. L. Seaton. 1954. A method for the extraction and assay of nucleic acid fragments in tissues. J. Am. Chem. Soc. **76**:2873-2877.

Eaton, N. R., and R. Caffrey. 1961. Effect of dihydrostreptomycin on *Escherichia coli*. J. Bacteriol. **81**:918-923.

Gale, E. F., and E. S. Taylor. 1947. The assimilation of amino acids by bacteria. 5. The action of penicillin in preventing the assimilation of glutamic acid by *Staphylococcus aureus*. J. Gen. Microbiol. **1**:314-326.

Garrod, L. P., and P. M. Waterworth. 1956. Behaviour *in vitro* of some new antistaphylococcal antibiotics. Brit. Med. J. **2**:61-65.

Hanawalt, P. 1959. Use of phosphorus-32 in microassay for nucleic acid synthesis in *Escherichia coli*. Science **130**:386-387.

Hancock, R. 1958. Protection of *Staphylococcus aureus* from some effects of penicillin by media of high osmotic pressure. Biochem. J. **70**:15P.

Jordan, D. C., and W. E. Inniss. 1959. Selective inhibition of ribonucleic acid synthesis in *Staphylococcus aureus* by vancomycin. Nature **184**:1894-1895.

Kirby, W. M. M., and C. L. Divelbiss. 1959. Vancomycin: clinical and laboratory studies. Antibiotics Ann. 1958-59, p. 107-117.

Park, J. T., and R. Hancock. 1960. A fractionation procedure for studies on the synthesis of cell-wall mucopeptide and of other polymers in cells of *Staphylococcus aureus*. J. Gen. Microbiol. **22**:249-258.

Park, J. T., and J. L. Strominger. 1957. Mode of action of penicillin. Science **125**:99-101.

Tsuboi, K. K., and T. D. Price. 1959. Isolation, detection and measure of microgram quantities of labelled tissue nucleotides. Arch. Biochem. Biophys. **81**:223-237.

Ziegler, D. W., R. N. Wolfe, and J. M. McGuire. 1956. Vancomycin, a new antibiotic. II. *In vitro* antibacterial studies. Antibiotics Ann. 1955-56, p. 612-622.

# 5-FLUOROURACIL AS AN INHIBITOR OF BACTERIAL CELL-WALL SYNTHESIS

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Fluorouracil is known to be metabolized like uracil in mammalian cells (Harbers, Chaudhuri, and Heidelberger, 1959) and in microorganisms (Cohen et al., 1958; Brockman, Davis, and Stutts, 1960). Fluorodeoxyuridylic acid, one of the products of such metabolism, is known to inhibit thymidylic acid synthetase (Cohen et al., 1958; Heidelberger et al., 1960) thus blocking deoxyribonucleic acid synthesis with consequent growth inhibitory effects. Other sites of inhibition have been recognized. For example, fluorouracil is known to be incorporated into various ribonucleic acid (RNA) fractions in place of uracil (Chaudhuri, Montag, and Heidelberger, 1958; Gros, *in press*); protein synthesis is affected, as evidenced by apparently specific alterations in certain enzyme activities in bacteria exposed to fluorouracil (Bussard et al., 1960; Naono and Gros, 1960). Possible sites of inhibition by fluorouracil also exist through interference with the synthesis or normal utilization of uridine diphosphate sugars. Peptide derivatives of certain uridine diphosphate sugars appear to play an important role in bacterial cell-wall synthesis and are known to accumulate in bacteria in the presence of penicillin, bacitracin, cycloserine, and novobiocin (Salton, 1960). Accumulations of uridine diphosphate muramic acid peptide, the compound that accumulates in penicillin-treated bacteria, or the fluorouracil-containing analogue of such a compound, have been described in fluorouracil-treated *Escherichia coli* and *Staphylococcus aureus* (Tomasz and Borek, 1960; Rogers and Perkins, 1960; Jacobs, Moorman, and Brockman, 1961). The results of the present study are in support of the view that, in addition to its other inhibitory effects, fluorouracil may also interfere with cell-wall synthesis in certain microorganisms.

## Materials and Methods

*S. aureus* 209P, designated here as Staph/O, was obtained from the American Type Culture Collection and was grown in Trypticase-soy-dextrose broth (Baltimore Biological Laboratory). Medium for growth inhibition studies was inoculated from 18-hr cultures in broth

and then distributed in 20-ml portions into standard taper 125-ml Erlenmeyer flasks fitted with side arms which were plugged with cotton. Each flask carried an 18 by 160 mm culture tube sealed to a ground-glass joint so that growth measurements could conveniently be made by inverting the flasks and determining the optical density at 660 mμ in a Bausch and Lomb Spectronic 20 colorimeter. The flasks were mechanically agitated in a water bath at 37 C to provide aerobic growth conditions. Inhibitors were added during the exponential-growth phase when the cultures reached an optical density of approximately 0.3 to 0.4. Similarly, 70-ml bacterial cultures in 300-ml Erlenmeyer flasks were incubated with radioactive tracers; hot aqueous alcoholic extracts of the cells were analyzed by chromatographic and radioautographic procedures in the manner previously described (Brockman et al., 1960). Two-dimensional descending paper chromatography of concentrated extracts was carried out using Whatman no. 1 filter paper; 72% aqueous phenol was used to develop the paper in the first dimension, and *n*-butanol-propionic acid-water (17:8.8:12; v/v) in the second dimension. One-dimensional descending chromatography, using Whatman 3MM paper with the ethanol-ammonium acetate (pH 7.5) solvent system described by Paladini and Leloir (1952), was particularly useful for separation of pyrimidine nucleotides and sugar derivatives and for confirmation of identity of such compounds. Paper electrophoresis was carried out in a Markham and Smith (1952) apparatus, using 0.05 M ammonium formate (pH 3.5) and 0.05 M sodium borate (pH 9) as buffers according to procedures described by Markham (1955). Radioactive areas of the papers were located by exposing the papers to X-ray film (Eastman Kodak, single emulsion, blue-sensitive) for 1 to 2 weeks.

The nonradioactive fluorouracil used in this study was a gift from Robert Duschinsky, Hoffmann-LaRoche; radioactive fluorouracil-2-$C^{14}$ and uracil-2-$C^{14}$ were obtained from the Isotopes Specialties Co., and uridine-2-$C^{14}$ was purchased from the New England Nuclear Corp. The following radioactive amino acids were obtained from the California Corporation for Biochemical Research: DL-alanine-2-$C^{14}$, DL-glutamic acid-3,4-$C^{14}$, and DL-lysine-2-$C^{14}$. Crystalline potassium penicillin G was used throughout this study. We are indebted to Kenneth Price, Bristol Laboratories, for a sample of 2,6-dimethoxyphenylpenicillin, and to H. M. Higgins, Eli Lilly and Co., and to Marvin Bachman and P. H. Hidy, Commercial Solvents Corp., for samples of cycloserine.

## Results

In a previous study (Jacobs et al., 1961) with *E. coli* ATCC 9637, it was observed that low concentrations of fluorouracil (5 μg/ml) caused extensive cell lysis about one generation time after addition

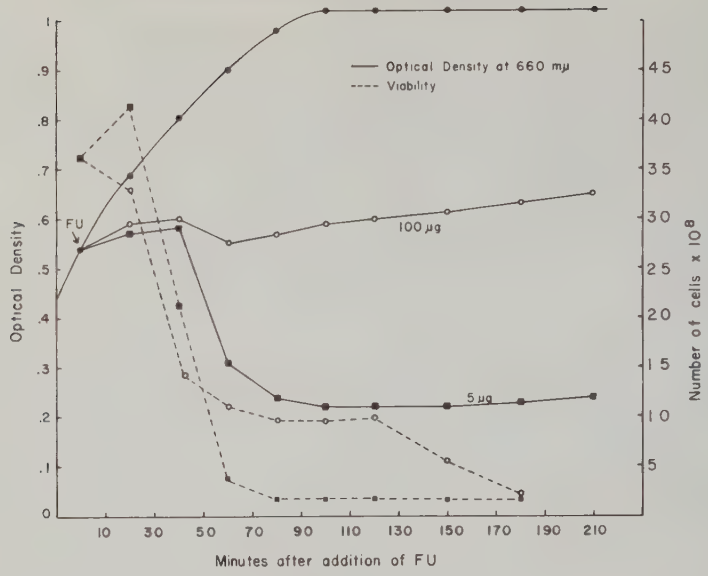


FIG. 1. *Effect of 5-fluorouracil on the growth and viability of Escherichia coli ATCC 9637.*

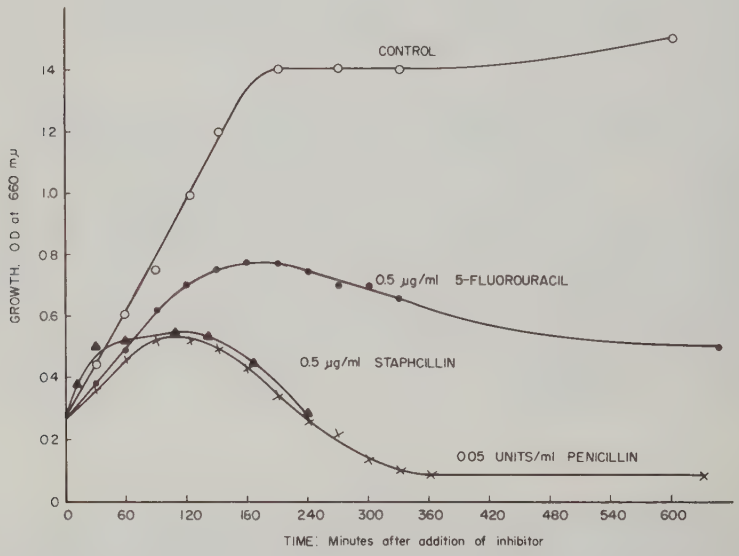


FIG. 2. *Effect of 5-fluorouracil, penicillin G, and 2,6-dimethoxyphenylpenicillin (Staphcillin) on the growth of Staphylococcus aureus 209P.*

of the inhibitor to exponentially growing cultures; higher concentrations (100 $\mu$g/ml) inhibited growth but did not cause pronounced lysis (Fig. 1). The effects of fluorouracil on Staph/O were not so striking as were those on *E. coli;* however, lysis followed exposure of Staph/O to low levels of fluorouracil (0.5 to 1 $\mu$g/ml) but not to higher levels of this inhibitor. Penicillin (0.05 unit/ml) and 2,6-dimethoxyphenylpenicillin (0.5 $\mu$g/ml) both produced lysis under the same conditions of rapid aerobic growth (Fig. 2).

Combinations of fluorouracil and penicillin were examined for their effect on Staph/O. As shown in Fig. 3, 0.01 $\mu$g/ml of fluorouracil in combination with $5 \times 10^{-4}$ unit/ml of penicillin caused almost as extensive lysis as did 0.05 unit/ml of penicillin alone. When tested singly at these low concentrations, neither fluorouracil nor penicillin caused inhibition of growth or produced cell lysis.

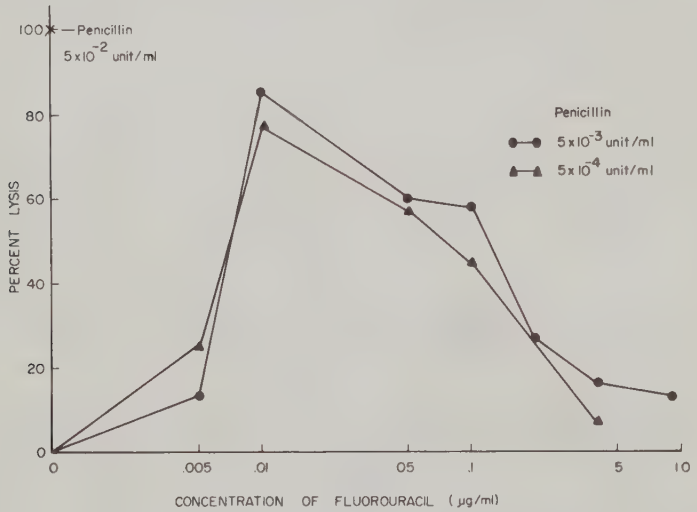


FIG. 3. *Lysis of Staphylococcus aureus 209P by combinations of 5-fluorouracil and penicillin G. Observed lysis is expressed as a percentage of the maximal decrease in optical density of the culture produced by 0.05 unit/ml of penicillin.*

An accumulation of Elson-Morgan hexosamine in fluorouracil-treated Staph/O could be demonstrated, using an adaptation of the procedure described by Strominger (1957). In agreement with the findings of Rogers and Perkins (1960), the amount of hexosamine extracted from the cells was observed to increase with increasing

fluorouracil concentration (Fig. 4); for comparison, the hexosamine accumulations in the presence of penicillin and cycloserine are also shown. The hexosamine accumulating in the presence of fluorouracil reacted with *p*-dimethylaminobenzaldehyde in the Elson-Morgan reaction to give a product with an ultraviolet absorption spectrum identical with that obtained from *N*-acetylglucosamine; maxima at 545 and 585 m$\mu$; a minimum at 565 m$\mu$. Uracil and uridine eliminated the fluorouracil-induced accumulation of hexosamine, and, as in the case of *E. coli*, these pyrimidines also prevented the inhibition of growth and lysis produced by fluorouracil. Thymine and thymidine also eliminated hexosamine accumulation and lysis caused by fluorouracil, but these agents did not completely overcome growth inhibition. This result is in agreement with other findings that thymine or thymidine alone cannot completely overcome the growth-inhibitory effects of fluorouracil in microorganisms (Cohen et al., 1958; Horowitz, Saukkonen, and Chargaff, 1960; Aronson, 1961).

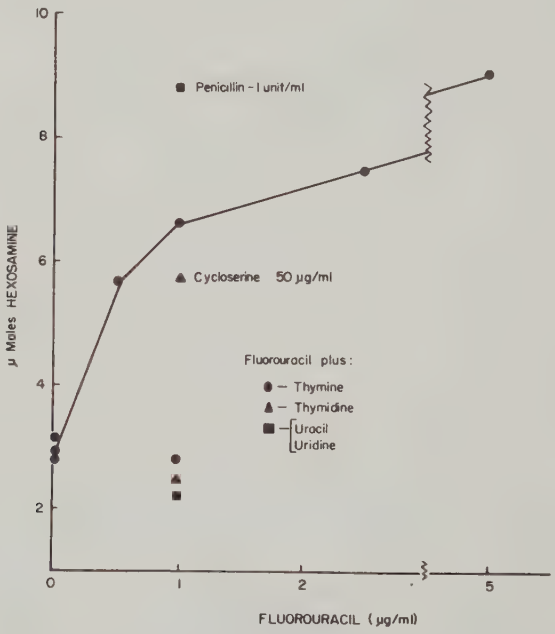


FIG. 4. *Accumulation of Elson-Morgan hexosamine in Staphylococcus aureus 209P 1 hr after exposure of the cultures to 5-fluorouracil. Hexosamine accumulation in the presence of penicillin and cycloserine are also indicated. Simultaneous addition of uracil (5 μ moles/ml) or of uridine, thymine, or thymidine (each at 0.5 μ moles/ml) eliminated hexosamine accumulation in cultures treated with fluorouracil (1 μg/ml). Hexosamine accumulation is expressed as moles of N-acetylglucosamine obtained by cold trichloroacetic acid extraction of the cells from 1 liter of culture medium.*

It is of interest that one fluorouracil-resistant strain of *S. aureus* (Staph/FU-1) exhibited significant cross-resistance to penicillin and 2,6-dimethoxyphenylpenicillin; a second strain (Staph/FU-2), although equally as resistant to fluorouracil as Staph/FU-1, was inhibited and lysed by the same concentrations of these antibiotics which produced similar effects in Staph/O (Fig. 5; Table 1).

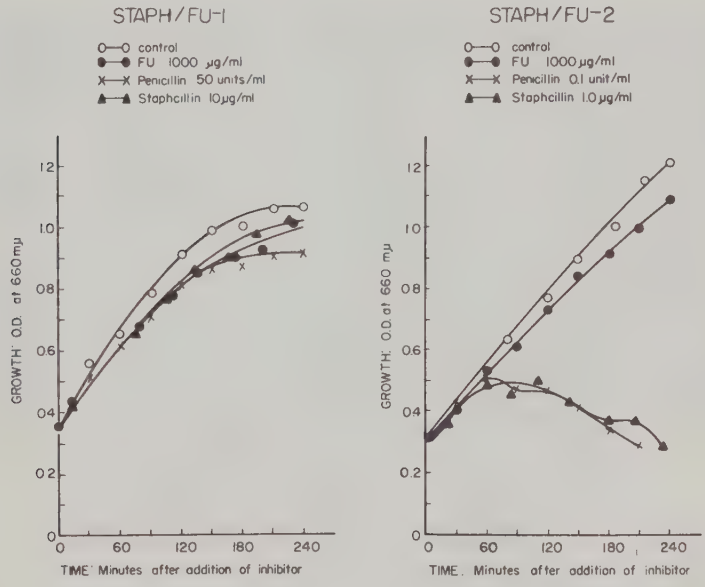


FIG. 5. *Comparison of the effects of 5-fluorouracil, penicillin, and 2,6-dimethoxyphenylpenicillin on the growth of two different fluorouracil-resistant strains of Staphylococcus aureus 209P.*

TABLE 1. *Effect of fluorouracil (FU), penicillin, and 2, 6-dimethoxyphenylpenicillin (Staphcillin) on growth of FU-sensitive and FU-resistant strains of Staphylococcus aureus 209P*

| Strain | Inhibitory concentrations | | |
|---|---|---|---|
| | FU | Penicillin | Staphcillin |
| | *µg/ml* | *units/ml* | *µg/ml* |
| Staph/O | 0.5* | 0.05* | 0.5* |
| Staph/FU-1 | >1000 | 10 | 100 |
| Staph/FU-2 | >1000 | 0.05* | 0.5* |

*Lysis occurred.

Results of a comparison of the metabolism of fluorouracil-2-$C^{14}$ in sensitive and fluorouracil-resistant *S. aureus* are shown in Fig. 6; it is evident that fluorouracil was extensively metabolized to fluorouridine and to nucleotide derivatives by Staph/O. In sharp contrast to this pattern of metabolism, the fluorouracil-resistant strains, represented in Fig. 6 by Staph/FU-1, failed to metabolize fluorouracil-2-$C^{14}$.

FIG. 6. *Comparison of the metabolism of 5-fluorouracil-2-$C^{14}$ in sensitive and fluorouracil-resistant Staphylococcus aureus 209P by chromatographic-radioautographic techniques. FU is 5-fluorouracil and FUR is 5-fluorouridine; FUDPX is described in the text (see also Table 2).*

Similar results were obtained in studies with fluorouracil-resistant *E. coli* (Brockman et al., 1960) and *Streptococcus faecalis* (Brockman and Sparks, 1960). Although not illustrated here, uracil-2-$C^{14}$ was extensively metabolized by Staph/O but not by the resistant strains; similarly, uridine-2-$C^{14}$ was converted to nucleotides by Staph/O but not by Staph/FU-1 or Staph/FU-2 (Fig. 7).

The radioactive material designated FUDPX (Fig. 6) is of particular interest since this same chromatographic area contained an Elson-Morgan positive component and was also radioactive when penicillin- or fluorouracil-inhibited Staph/O was incubated with radioactive alanine, glutamic acid, or lysine. Results of further analysis by means of paper chromatography and electrophoresis are summarized in Table 2. These data show that penicillin treatment of Staph/O in the presence of radioactive alanine, glutamic acid, or lysine gave rise to a radioactive compound derived from each of these substrates; this compound was characterized by similar migration behavior in two-dimensional chromatography and by paper

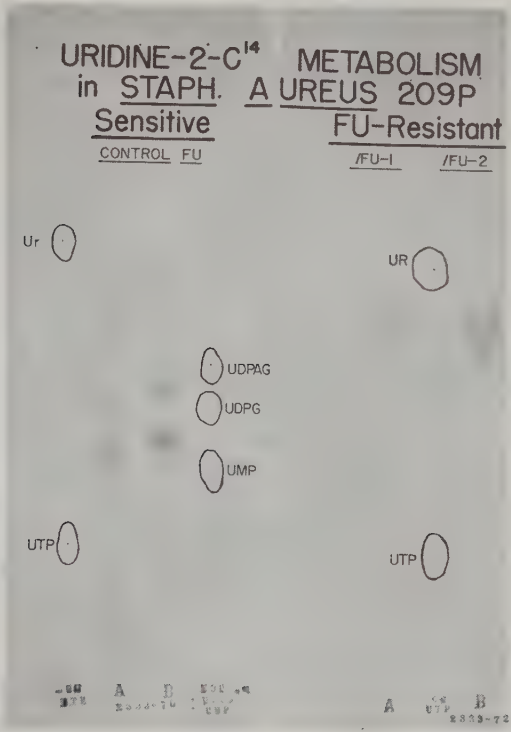


FIG. 7. *Comparison of the metabolism of uridine-2-$C^{14}$ by fluorouracil-sensitive and fluorouracil-resistant strains of Staphylococcus aureus. Abbreviations: Ur, uridine; UMP, uridine-5'-phosphate; UTP, uridine-5'-triphosphate; UDPG, uridine-5'-diphosphate glucose; UDPAG, uridine-5'-diphosphate N-acetylglucosamine.*

TABLE 2. *Characterization of "peptide" formed by Staphylococcus aureus 209P in the presence of penicillin or fluorouracil*

| Substrate | Two-dimensional chromatography* ($R_F$ value × 100) | | | Relative electrophoretic migration† | |
|---|---|---|---|---|---|
| | Reference known‡ | Penicillin | Fluorouracil | Penicillin | Fluorouracil |
| DL-Alanine-2-$C^{14}$ | 62:32 | 28:5 | 32:4 | 79 | 79 |
| DL-Glutamic acid-3, 4-$C^{14}$ | 44:26 | 28:2 | 30:2 | 83 | 81 |
| DL-Lysine-2-$C^{14}$ | 40:11 | 29:4 | 30:4 | 81 | 81 |
| 5-Fluorouracil-2-$C^{14}$ | 64:62 | | 31:3 | | 79 |

*Chromatography in phenol-water and *n*-butanol-propionic acid-water.

†Migration distance (cm) relative to uridine-5'-phosphate (as 100) in 0.05 M ammonium formate buffer (pH 3.5) at a voltage gradient of 18 v/cm on Whatman no. 3 MM paper for 2 hr.

‡$R_F$ values (× 100) of the substrates. The numbers preceding the colon give the $R_F$ value in the first dimension (phenol-water); the numbers following the colon give the $R_F$ in the second dimension (*n*-butanol-propionic acid-water).

electrophoresis. Fluorouracil treatment of Staph/O in the presence of these substrates gave rise to a compound which behaved identically with that formed in the presence of penicillin.

## Discussion

Fluorouracil-induced fragility of bacterial cells (Tomasz and Borek, 1959) and the increased survival of fluorouracil-treated cells in the presence of high concentrations of sucrose attracted attention to the possibility that this uracil analogue might be affecting cell-wall synthesis. When the rather nonspecific phenomenon of cell lysis is coupled with observations on the accumulation of hexosamine in the presence of fluorouracil, and more specifically, on the formation of fluorouridine diphosphate muramic acid peptide, then the observed fluorouracil-induced lysis takes on greater significance.

It is of interest that low concentrations of fluorouracil in combination with quite low levels of penicillin gave maximal lysis and that higher levels of fluorouracil decreased the extent of cell lysis. To demonstrate lysis in the present experiments, it was apparently necessary that the bacterial cultures be in a phase of rapid growth and hence of rapid synthesis of new cell-wall material at the time the inhibitor was added. It appears likely that the higher levels of fluorouracil may decrease the extent of lysis by slowing the growth rate of the cells.

The results of Rogers and Perkins (1960) suggest that the fluorouracil analogue of the muramic acid peptide is not itself readily utilizable for cell-wall synthesis. It has been postulated that penicillin interfered with the introduction of the muramic acid peptide unit into the cell-wall structure (Strominger, 1960). The observation that concentrations of fluorouracil and penicillin which, alone, were subinhibitory but when used in combination produced extensive lysis of Staph/O can be interpreted as an indication that these agents act at different sites or by different mechanisms in interfering with cell-wall synthesis. The isolation of a fluorouracil-resistant mutant exhibiting cross-resistance to penicillin was, therefore, an unexpected result. A second fluorouracil-resistant mutant was subsequently isolated and found to be equally as sensitive to penicillin as was Staph/O. The mechanism by which resistance to penicillin arose during the isolation of the first fluorouracil-resistant mutant is not known, but the observed cross-resistance of Staph/FU-1 to 2,6-dimethoxyphenylpenicillin suggests that increased penicillinase production is probably not a factor.

A comparison of the metabolism of pyrimidines in the fluorouracil-sensitive and fluorouracil-resistant organisms revealed that both resistant mutants had lost the capacity to metabolize uracil, fluorouracil, and uridine to nucleotides, whereas the parent sensitive

strain retained this capacity. Loss of capacity to form nucleotide derivatives accompanying the development of resistance to pyrimidine and purine analogues has been observed in a number of biological systems (Anderson and Law, 1960). Loss of capacity to form fluorouracil nucleotides is a particularly effective resistance mechanism, since fluorodeoxyuridine-5'-phosphate is the form of the inhibitor that is active in blocking thymidylic acid synthesis and since fluorouridine-5'-phosphates must be formed before the analogue can be incorporated into RNA or into glycosyl nucleotide derivatives. Prevention of fluorouracil inhibition by uracil and uridine is readily explainable in terms of the known pathways by which uracil is metabolized to nucleotides:

(1) Uracil + 5-phosphoribosyl-1-pyrophosphate → uridylic acid + pyrophosphate
(2) Uracil + ribose-1-phosphate → uridine + phosphate
(3) Uridine + adenosine triphosphate → uridylic acid + adenosine diphosphate.

Uracil competes with fluorouracil for the enzymes and substrates of reactions 1 and 2. On an equimolar basis, uridine was observed to be more effective than uracil in preventing fluorouracil inhibition of Staph/O, suggesting that perhaps reactions 2 and 3, rather than reaction 1, represent the major pathways for metabolism of uracil to uridylic acid in this organism. The observation that thymine and thymidine were also capable of preventing fluorouracil-induced lysis of *S. aureus* and of *E. coli* (Jacobs et al., 1961) deserves further study. It may be that thymine nucleotide derivatives also play a role in bacterial cell-wall synthesis.

## Acknowledgments

The authors are indebted to Sigrid B. Jacobs for the *E. coli* experiments described in Fig. 1. We are grateful to Robert F. Pittillo for helpful discussions of aspects of this work. The work was supported by grants from the Charles F. Kettering Foundation, the Alfred P. Sloan Foundation, and the Southern Research Institute.

## Literature Cited

Anderson, E. P., and L. W. Law. 1960. Biochemistry of cancer. Ann. Rev. Biochem. **29**:577-608.

Aronson, A. 1961. The inhibition of bacteriophage protein synthesis by 5-fluorouracil. Biochim. et Biophys. Acta **49**:89-97.

Brockman, R. W., J. M. Davis, and P. Stutts. 1960. Metabolism of uracil and 5-fluorouracil by drug-resistant bacteria. Biochim. et Biophys. Acta **40**:22-32.

Brockman, R. W., and M. C. Sparks. 1960. Metabolism of pyrimidines and 5-fluoropyrimidines by drug-sensitive and drug-resistant *Streptococcus faecalis*. Bacteriol. Proc., p.71.

Bussard, A., S. Naono, F. Gros, and J. Monod. 1960. Effets d'un analogue de l'uracile sur les propriétes d'une, protéine enzymatique synthétisée en sa précence. Compt. rend. **250**: 4049-4051.

Chaudhuri, N. K., B. J. Montag, and C. Heidelberger. 1958. Studies on fluorinated pyrimidines. III. The metabolism of 5-fluorouracil-2-$C^{14}$ and 5-fluoroorotic-2-$C^{14}$ acid *in vivo*. Cancer Research **18**:318-328.

Cohen, S. S., J. G. Flaks, H. D. Barner, M. R. Loeb, and J. Lichtenstein. 1958. The mode of action of 5-fluorouracil and its derivatives. Proc. Natl. Acad. Sci. U. S. **44**:1004-1012.

Harbers, E., N. K. Chaudhuri, and C. Heidelberger. 1959. Studies on fluorinated pyrimidines. VIII. Further biochemical studies. J. Biol. Chem. **234**:1255-1262.

Heidelberger, C., G. Kaldor, K. L. Mukherjee, and P. B. Danneberg. 1960. Studies on fluorinated pyrimidines. XI. *In vitro* studies on tumor resistance. Cancer Research **20**:903-909.

Horowitz, J., J. J. Saukkonen, and E. Chargaff. 1960. Effects of fluoropyrimidines on the synthesis of bacterial proteins and nucleic acids. J. Biol. Chem. **235**:3266-3272.

Jacobs, S. B., J. A. Moorman, and R. W. Brockman. 1961. Evidence for 5-fluorouracil inhibition of cell wall synthesis in *Escherichia coli* and *Staphylococcus aureus*. Bacteriol. Proc., p. 190.

Markham, R. 1955. Nucleic acids, their components and related compounds, p.246-304. *In* K. Paech and M. V. Tracey [ed.], Modern methods of plant analysis vol. 4, Springer-Verlap, Berlin.

Markham, R., and J. D. Smith. 1952. The structure of ribonucleic acids. I. Cyclic nucleotides produced by ribonuclease and by alkaline hydrolysis. Biochem. J. **52**:552-557.

Naono, S., and F. Gros. 1960. Synthése par *E. coli* d'une phosphatase modifée en présence d'un analogue pyrimidique. Compt. rend. **250**:3889-3891.

Paladini, A. C., and L. F. Leloir. 1952. Studies on uridine diphosphate glucose. Biochem. J. **51**:426-430.

Rogers, H. J., and H. R. Perkins. 1960. 5-Fluorouracil and mucopeptide biosynthesis by *Staphylococcus aureus*. Biochem. J. **77**:448-459.

Salton, M. R. J. 1960. Microbial cell walls. CIBA Lectures in microbial biochemistry. John Wiley and Sons, Inc., New York.

Strominger, J. L. 1957. Microbial uridine-5'-pyrophosphate *N*-acetylaminosugar compounds. I. Biology of the penicillin-induced accumulation. J. Biol. Chem. **224**:509-523.

Strominger, J. L. 1960. Mononucleotide acid anhydrides and related compounds as intermediates in metabolic reactions. Physiol. Rev. **40**:55-111.

Tomasz, A., and E. Borek. 1959. An early phase in the bactericidal action of 5-fluorouracil on *E. coli* K-12: osmotic imbalance. Proc. Natl. Acad. Sci. U. S. **45**:929-932.

Tomasz, A., and E. Borek. 1960. The mechanism of bacterial fragility produced by 5-fluorouracil: the accumulation of cell wall precursors. Proc. Natl. Acad. Sci. U. S. **46**:324-327.

# ACTION OF CYCLOHEXIMIDE ON ANIMAL CELLS

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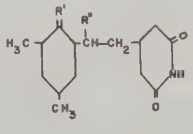
Cycloheximide (Acti-dione, The Upjohn Co.) is an antibiotic produced by *Streptomyces griseus*. It inhibits growth of many yeasts and molds (Whiffen, 1948), protozoa (Loefer and Matney, 1952), and algae (Palmer and Maloney, 1955), and is toxic for certain higher plants (Ford, Klomparens, and Hamner, 1958), animals (Ford and Leach, 1948), and mammalian cells in continuous culture (Smith et al., 1960). Currently, cycloheximide is used primarily to control plant mycoses; however, antibiotics having this general structure (Fig. 1) possess significant antitumor activity.




| | R' | R" |
|---|---|---|
| CYCLOHEXIMIDE | =O | -OH |
| C· ACETATE | =O | $-OCOCH_3$ |
| C·SEMICARBAZONE | $=N\cdot NHCONH_2$ | -OH |

FIG. 1. *Structural formulae of cycloheximide, cycloheximide acetate, and cycloheximide semicarbazone.*

Cycloheximide in fungistatic concentrations has little effect on the energy-generating process of *Saccharomyces carlsbergensis*, but does inhibit protein and nucleic acid syntheses (Kerridge, 1958). Protein and deoxyribonucleic acid (DNA) syntheses are more susceptible to the action of the drug than is ribonucleic acid (RNA) synthesis. In addition, cycloheximide is reported to inhibit the glutamine synthetase enzyme of *S. pastorianus* (Coursen and Sisler, 1960) and the enzymes alcohol dehydrogenase and lactic dehydrogenase (Latuasan and Berends, 1958). This report is concerned with our studies on the effect of cycloheximide on the protozoon *Tetrahymena* and on an established line of human esophageal epithelial cells. In both animal cells, DNA synthesis was more susceptible to the antibiotic than

was protein synthesis, RNA synthesis, or energy generation and transfer.

## Materials and Methods

Cells of human esophageal epithelium (Minn. EE 55-12-1) have been subcultured for 6 years as a stable line, after establishment from tissue of a male infant with a tracheoesophageal fistula (Syverton and McLaren, 1957). A one-membered culture of *T. pyriformis* strain B was generously supplied by N. S. Kerr, Department of Zoology, University of Minnesota. Medium for growth and procedures for subculture of parental, uncloned EE cells have been described previously (Bradley and Syverton, 1960). The growth medium was supplemented with vitamins and essential and nonessential amino acids (Eagle, 1959). *Tetrahymena* was cultivated in a medium containing 5 g of Difco peptone, 3 g of Difco yeast extract, and 1 liter of deionized water. Growth was measured by direct hemocytometer counts of formalin-killed Tetrahymena and trypsin-dispersed EE cells. Stock cultures were checked frequently for presence of bacteria or pleuropneumonia-like organisms. No antibiotic other than cycloheximide was added to the growth media. Cycloheximide, cycloheximide acetate, and cycloheximide semicarbazone (courtesy of The Upjohn Co.) were dissolved in isotonic NaCl-KCl solution at pH 5 and sterilized by autoclaving at 121 C for 15 min or by filtration through Selas or Millipore filters. Solutions or suspensions of other test substances were sterilized by autoclaving, filtration, or by treatment with absolute ethanol, according to the nature of the compound.

Protein content of EE cultures, washed free of serum protein and lysed in distilled water, was measured by the biuret reaction (Levin and Brauer, 1951). Color intensity was read in a Bausch and Lomb Spectronic 20 colorimeter at 540 mμ. Crystallized bovine plasma albumin (Armour) was used as the reference standard. Protein synthesis in *Tetrahymena* was followed by both the biuret method and the Koch-McMeekin Nessler method for total nitrogen. Nucleic acid content of samples of *Tetrahymena* that were harvested, washed with 50% ethanol, dried, and weighed was determined colorimetrically. The nucleic acids were extracted with trichloroacetic acid (Schneider, 1945); RNA was estimated by the orcinol reaction for ribose (Ceriotti, 1955), and DNA by the indole reaction for deoxyribose (Ceriotti, 1952). Salmon-sperm DNA and yeast RNA were used as reference standards. Oxygen consumption and aerobic carbon dioxide evolution were measured manometrically in a Warburg apparatus at 22 C. Dissolved carbon dioxide was released from the neutral suspending medium by addition of acid. The incidence of mitotic figures was determined for EE cells, fixed in Zenker's fluid and stained with Harris hematoxylin.

## Results

Cycloheximide at concentrations of 1 μg/ml and below inhibited significantly growth of EE cells and *Tetrahymena;* the extent of inhibition was dependent upon inoculum size (Fig. 2). The greatest relative difference in number of EE cells in antibiotic-treated and control cultures occurred 48 to 72 hr after addition of cycloheximide; with *Tetrahymena,* maximal inhibition was expressed 30 to 48 hr after challenge. Both EE cells and *Tetrahymena* began to recover from the toxic effects of 3 μg antibiotic/ml or less after 3 to 4 days exposure, and achieved normal population densities by 10 to 12 days. These cells were fully sensitive to cycloheximide upon subsequent testing. With 10 μg antibiotic/ml, the majority of the cells were killed during the first 24 hr of exposure; the remaining cells did not grow out in the presence of the drug, but were able to generate new populations when transferred to antibiotic-free medium.

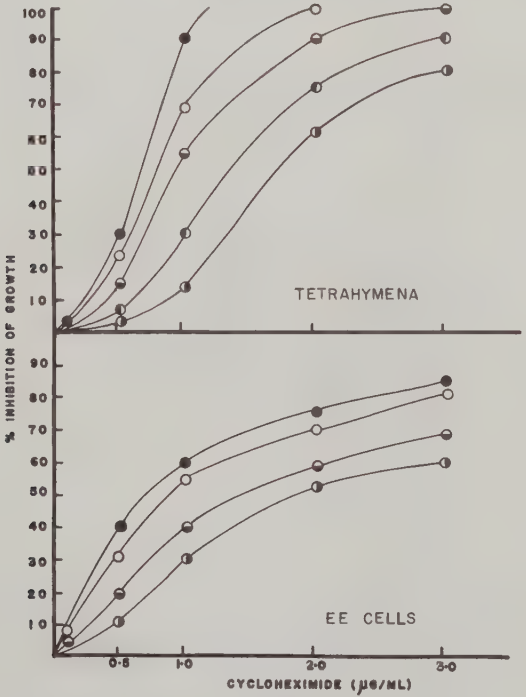



FIG. 2. *Effect of inoculum size on action of cycloheximide. Peptone-yeast extract broth was inoculated with* $2\times10^2$ (●), $1\times10^3$ (O), $2\times10^3$ (◒), $1\times10^4$ (◐), *or* $2\times10^4$ (◑) *Tetrahymena/ml; cell counts were made after 48 hr incubation at 21 C. EE cells were added to an initial population density of* $2.5\times10^4$ (●), $5\times10^4$ (O), $1\times10^5$ (◒), *or* $2\times10^5$ (◐) *per ml; cell counts were made after 60 hr incubation at 37 C.*

The optimal temperature for growth of *Tetrahymena* was 21 C, although it grew slowly at 10 and 37 C. Maximal inhibition of growth occurred at 10 to 25 C; at 30 to 37 C, 1 μg cycloheximide/ml was significantly but only weakly toxic (Fig. 3). The reduced antibiotic activity was not the result of thermal inactivation of the drug. With EE cells, the optimal growth temperature was 35 to 37 C; elevated temperatures killed the cells, whereas lower temperatures allowed the EE cells to persist but not to reproduce. EE cells exposed to 10 to 15 μg cycloheximide/ml for 4 to 6 days at 22 C were still fully viable, and initiated growth promptly when transferred to antibiotic-free medium at 37 C. *Tetrahymena* grew best between pH 5 and 7; definite but poor growth occurred at pH 3.5 and 10. Cycloheximide manifested greatest toxicity at pH 5 and least toxicity at pH 10 (Fig. 3). The antibiotic was progressively degraded in neutral and alkaline solutions at incubation temperatures.

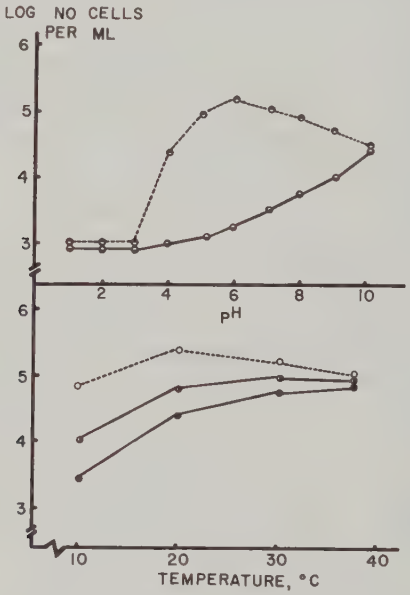



FIG. 3. *Effect of temperature and pH on action of cycloheximide. Tetrahymena cultures, challenged with no antibiotic (O), 1 μg/ml (◑), and 2 μg/ml (●), were scored after 4 days incubation at the indicated temperature. Protozoan cultures, exposed to no antibiotic (◓) or 3 μg/ml (◒), were counted after 2 days incubation at the indicated pH.*

Various organic and inorganic compounds were tested for ability to protect *Tetrahymena* from cycloheximide toxicity (Tables 1 and 2). Similarly, inorganic salts, vitamins, and amino acids were tried with EE cultures. Activated charcoal was the only substance that reduced

TABLE 1. *Compounds unable to protect against cycloheximide activity**

| Inorganic salts | Amino acids | Vitamins |
|---|---|---|
| $AlCl_3$ | Alanine | Biotin |
| $CaCl_2$ | Arginine | Choline |
| $Ca(NO_3)_2$ | Asparagine | Folic acid |
| $CdCl_2$ | Aspartic acid | Nicotinamide |
| $CuSO_4$ | Cysteine | Pantothenic acid |
| $FeCl_3$ | Cystine | Pyridoxal |
| $FeSO_4$ | Glutamic acid | Thiamine |
| KCl | Glutamine | Riboflavin |
| LiCl | Histidine | Myo-inositol |
| $MgCl_2$ | Isoleucine | DL-Tocopherol |
| $MnSO_4$ | Leucine | *p*-Aminobenzoic acid |
| NaCl | Lysine | Vitamin K |
| $NiSO_4 \cdot 6H_2O$ | Methionine | Vitamin $B_{12}$ |
| RbCl | Phenylalanine | $\beta$-Carotene |
| $UO_2(C_2H_3O_2)_2 \cdot 2H_2O$ | Proline | Ascorbic acid |
| $ZnCl_2$ | Serine | $\alpha$-Lipoic acid |
| $NH_4Cl$ | Threonine | |
| | Tryptophan | |
| | Tyrosine | |
| | Glycine | |

*Inorganic salts were tested in a concentration range of 1 to 100 $\mu$g/ml; organic compounds in a range of 10 to 1000 $\mu$g/ml. Cycloheximide was added to *Tetrahymena* cultures or EE cells to give a final concentration of 2 $\mu$g/ml.

TABLE 2. *Compounds unable to protect against cycloheximide activity**

| Nucleic acid derivatives | Sterols | Miscellaneous |
|---|---|---|
| Adenine | Estradiol | Ethanol |
| Guanine | Progesterone | Diphosphopyridine nucleotide |
| Cytosine | Sitosterol | Imidazole |
| Uracil | Cholesterol | Ribose |
| Thymine | Stigmasterol | Linoleic acid |
| Orotic acid | | Linolenic acid |
| Guanidine·HCl | | Sodium pyruvate |
| Deoxyadenosine | | Succinic acid |
| Deoxyadenylic acid | | Sodium acetate |
| Deoxycytidine | | Sodium citrate |
| Deoxycytidylic acid | | Ethylenediaminetetraacetic acid |
| Deoxyguanosine | | Margaric acid |
| Deoxyguanylate | | Sodium deoxycholate |
| Thymidylic acid | | Thioglycollic acid |
| Nucleinic acid | | Urea |
| | | Caproic acid |
| | | Glucose |

*Procedure same as in Table 1.

the power of the antibiotic to suppress growth. Cycloheximide was merely adsorbed from the medium by the charcoal. Cycloheximide acetate and cycloheximide semicarbazone inhibited EE cells and *Tetrahymena* when added at concentrations of 10 to 25 μg/ml or greater. Suppression of growth by mixtures of cycloheximide and its derivatives was cumulative. The final population densities of *Tetrahymena* cultures exposed to 2 μg cycloheximide/ml or 25 μg cycloheximide semicarbazone/ml, for example, were 50% less than those of untreated cultures. The two agents in combination suppressed growth by 75%.

Protein and RNA syntheses by *Tetrahymena* cells in the exponential phase of growth continued normally for 4 hr after addition of 2.5 μg of cycloheximide/ml. Subsequently, formation of protein and RNA was retarded, but was not halted completely for many hours. The RNA content of individual cells declined somewhat after 10 hr. Synthesis of DNA was retarded immediately upon exposure to the antibiotic, and ceased completely within a few hours. The DNA content of individual cells dropped steadily during the experiment (Fig. 4). EE cells challenged with 0.5 μg cycloheximide/ml continued

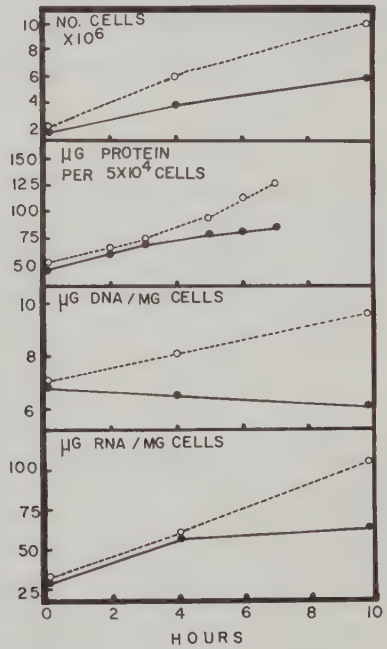



FIG. 4. *Effect of 2.5 μg cycloheximide/ml on reproduction and synthesis of nucleic acids and protein by Tetrahymena. Treated (solid lines) and untreated (dotted lines) cultures were incubated in peptone-yeast extract broth.*

to synthesize protein at the normal rate for 15 hr. Cultures exposed to the antibiotic for 60 hr contained 240 μg protein, whereas control cultures contained 600 μg. Oxygen consumption and aerobic carbon dioxide evolution by $5 \times 10^5$ *Tetrahymena* cells in growth medium was not inhibited by as much as 8 μg antibiotic/ml. Moreover, the protozoa remained actively motile in all tested drug concentrations.

The nuclei of EE cells treated with 2 μg cycloheximide/ml for 48 hr were pycnotic and the cytoplasm vacuolated. With 5 μg antibiotic/ml, the cytoplasm of many cells retracted from their nuclei, and nuclei without any apparent cytoplasm were observed occasionally. In control cultures, 35 mitotic figures were seen per 1,000 cells; with 0.1 μg cycloheximide/ml, there were 19 mitotic figures per 1,000 cells; with 1.0 μg/ml, 9; no mitotic figures were observed in cultures treated with 2 and 5 μg antibiotic/ml. In control cultures, all stages of mitosis were present, but only interphase and prophase were found in treated cultures.

## Discussion

Cycloheximide profoundly affects growth and metabolism of a great diversity of cells. The primary mechanism of drug action, however, seems to be the same for all susceptible organisms despite their structural and organizational disparity. Cycloheximide is lethal for metabolically active cells only in concentrations 100-fold higher than the minimal growth-inhibiting dose, and does not injure metabolically inactive cells. DNA synthesis is one of the first physiological activities affected by the antibiotic, both in *S. carlsbergensis* (Kerridge, 1958) and *Tetrahymena*. Moreover, 5 μg cycloheximide/ml induces aberrations in mitosis of onion root tip cells. Some event in interphase or early prophase is apparently inhibited (Hawthorne and Wilson, 1952). The effects of cycloheximide on nuclear division may be ascribed to interference with DNA synthesis, which takes place during interphase. The glutarimide moiety of the antibiotic structurally resembles the pyrimidine skeleton and can conceivably act as an analogue. This hypothesis is consistent with the finding that substitution on the cyclohexyl moiety does not destroy the antibiotic activity of the molecule. Free pyrimidines, nucleosides, and nucleotides, however, do not protect *Tetrahymena* treated with cycloheximide. Energy generation and transfer seem to be intact, because the protozoa remain actively motile for days in strongly growth-inhibiting concentrations of the drug.

Induced enzyme synthesis by *S. pastorianus* is prevented by fungistatic levels of cycloheximide. Induced enzyme formation is generally assumed not to require active participation by DNA, and suppression of the adaptive process is usually attributed to effects on protein synthesis, RNA synthesis, or energy transfer. Certainly cycloheximide affects the first two physiological processes, but seemingly

secondarily. The antibiotic may interfere with the function of deoxyribonucleotides located in the cytoplasm or with genetic material actively directing certain biosyntheses. Active intervention of DNA in metabolism is entirely plausible; for example, crystallized preparations of lactic dehydrogenase contain poly-deoxyribonucleotide (Appleby and Morton, 1959). If DNA is necessary for harmonious synthesis or activity of lactic dehydrogenase, the reported inhibition of this enzyme by cycloheximide (Latuasan and Berends, 1958) may be ascribed to the drug's proposed action on pyrimidine metabolism and poly-deoxyribonucleotide synthesis.

## Acknowledgments

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## Literature Cited

Appleby, C. A., and R. K. Morton. 1959. Lactic dehydrogenase and cytochrome $b_2$ of baker's yeast. Biochem. J. **73**:539-550.

Bradley, S. G., and J. T. Syverton. 1960. Inheritance of capability of human esophageal epithelial cells to grow with diverse carbohydrates. Proc. Soc. Exptl. Biol. Med. **103**:215-221.

Ceriotti, G. 1952. A microchemical determination of deoxyribonucleic acid. J. Biol. Chem. **198**: 297-303.

Ceriotti, G. 1955. Determination of nucleic acid in animal tissue. J. Biol. Chem. **214**:59-70.

Coursen, B. W., and H. D. Sisler. 1960. Effect of the antibiotic, cycloheximide, on the metabolism and growth of *Saccharomyces pastorianus*. Am. J. Botany **47**:541-549.

Eagle, H. 1959. Amino acid metabolism in mammalian cell cultures. Science. **130**:432-437.

Ford, J. H., W. Klomparens, and C. L. Hamner. 1958. Cycloheximide (Acti-dione) and its agricultural uses. Plant Disease Reptr. **42**:680-695.

Ford, J. H., and B. E. Leach. 1948. Acti-dione, an antibiotic from *Streptomyces griseus*. J. Am. Chem. Soc. **70**:1223-1225.

Hawthorne, M., and G. B. Wilson. 1952. The cytological effects of the antibiotic Acti-dione. Cytologia **17**:71-85.

Kerridge, D. 1958. The effect of Acti-dione and other antifungal agents on protein and nucleic acid synthesis in *Saccharomyces carlsbergensis*. J. Gen. Microbiol. **19**:497-506.

Levin, R., and R. W. Brauer. 1951. The biuret reaction for the determination of proteins—an improved reagent and its applications. J. Lab. Clin. Med. **38**:474.

Latuasan, H. E., and W. Berends. 1958. The action mechanisms of Acti-dione. Rec. trav. chim. **77**:416-422.

Loefer, J. B., and T. S. Matney. 1952. Growth inhibition of free living protozoa by Acti-dione. Physiol. Zool. **25**:272-276.

Palmer, C., and T. E. Maloney. 1955. Preliminary screening for potential algicides. Ohio J. Sci. **55**:1-8.

Schneider, W. C. 1945. Phosphorous compounds in animal tissues. I. Extraction and estimation of deoxypentose nucleic acid and of pentose nucleic acid. J. Biol. Chem. **161**:293-303.

Smith, C. G., W. L. Lummis, and J. E. Grady. 1960. Studies on the mode of action of streptovitacin A. Cancer Research **20**:1394-1398.

Syverton, J. T., and L. C. McLaren. 1957. Human cells in continuous culture. I. Derivation of cell strains from esophagus, palate, liver and lung. Cancer Research **17**:923-926.

Whiffen, A. J. 1948. The production, assay, and activity of Acti-dione, an antibiotic from *Streptomyces griseus*. J. Bacteriol. **56**:283-291.

# FURTHER STUDIES ON THE ANTIMICROBIAL NATURE OF ACTION OF AZASERINE

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Azaserine, functioning as a glutamine antagonist (Buchanan et al., 1957), inhibits a variety of glutamine-requiring biosynthetic reactions in both mammalian and bacterial cells. Some of these reactions are involved in purine metabolism: i.e., the amidination of formylglycinamide ribonucleotide to yield formylglycinamidine ribonucleotide (Hartman, Levenberg, and Buchanan, 1955; Tomisek, Kelly, and Skipper, 1956; Bennett, Schabel, and Skipper, 1956); the amination of 5-phosphoribosyl pyrophosphate to yield phosphoribosylamine (Buchanan et al., 1957); and the amination of xanthylic acid to yield guanylic acid (Abrams and Bentley, 1959). In bacteria, additional biosynthetic reactions are azaserine-sensitive: i.e., the conversion of shikimic acid-5-phosphate to anthranilic acid (Srinivasan, 1959) and the amination of the nicotinic acid moiety of di- and triphosphopyridine nucleotides to yield the corresponding nicotinamide pyridine nucleotides (Preiss and Handler, 1958).

The evidence indicates that in bacteria the inhibition of *de novo* purine biosynthesis is probably the prime site of azaserine attack, a thesis that implies that azaserine-inhibited cells die as a consequence of purine starvation. Some of the observations in bacterial systems cannot be explained on the basis of this premise. Inhibition of microbial growth by azaserine is best reversed by amino acids (Reilly, 1954); phenylalanine, tyrosine, and glutamine are effective. Furthermore, histidine in the presence of low concentrations of phenylalanine competitively reverses azaserine inhibition of proliferating *Escherichia coli* cells to a degree far in excess of that of glutamine (Pittillo, 1961). At best, inhibition caused by only threshold concentrations of azaserine can be reversed by purines (Bennett et al., 1956; Kaplan, Reilly, and Stock, 1959; Pittillo, 1961). Moreover, azaserine inhibition of *de novo* purine biosynthesis in *E. coli* is reversed by phenylalanine not only in actively growing cells but also in cell-free preparations obtained by ultrasonic procedures (Tomisek, Reid, and Skipper, 1959), the latter observation obviating the possibility that aromatic amino acid reversal of azaserine action is a function of some nonspecific effect, such as interference with

either the penetration of the antibiotic through the cell membrane or the absorption of azaserine by a cell-wall component.

These observations appear to indicate either that the first (most sensitive) growth-essential reaction or reactions in *E. coli* inhibited by azaserine are not in the currently defined areas of purine synthesis or that phenylalanine plays some heretofore unrecognized role in bacterial purine metabolism. The purpose of this communication is to present results to clarify this problem.

## Materials and Methods

*Microorganisms and conditions.* *E. coli* was grown on a synthetic glucose-salts medium (Anderson, 1946). All auxotrophic strains were grown on this medium supplemented with the required metabolite in suitable concentrations. Agar (2%) was added to this medium for some of the experiments. *E. coli* M83-5 (phenylalanine-requiring) was obtained from Bernard D. Davis. Purine-requiring *E. coli* B96 (Gots and Love, 1954) was originally obtained from J. S. Gots.

*Inhibition-reversal studies.* The spread-plate procedure was used to determine the effect of metabolites on inhibitor action (Foster and Pittillo, 1953). The minimal inhibitory concentration of inhibitor is defined as the lowest concentration completely preventing the appearance of colonies to the unaided eye. "Reversal" is defined as the development of colonies from all of the cells in the inoculum as a result of the incorporation of a material in the medium containing an otherwise inhibitory concentration of inhibitor.

*Experiments with liquid medium.* The effect of metabolites on the growth of the purine- or amino acid-requiring strains of *E. coli* was determined by culturing the bacteria in Anderson's synthetic glucose-salts medium supplemented with the essential growth requirement and the metabolite under investigation. Tubes containing 10 ml of medium were inoculated with 0.05 ml of a thrice-washed 18-hr culture suspension (turbidity adjusted to 22% light transmittance on a Bausch and Lomb Spectronic 20 spectrophotometer adjusted to 660 mμ and equipped with an infrared transmitting filter). The culture tubes were incubated in water baths at 37 C and were shaken each hour at the time of turbidity determinations.

*Metabolites tested for inhibitor-reversing activity.* Initial work consisted of individually testing the following materials for inhibitor-reversing activity: glycine, serine, histidine, alanine, threonine, leucine, isoleucine, sarcosine, valine, α-aminobutyric acid, aspartic acid, glutamic acid, glutamine, lysine, arginine, ornithine, citrulline, cystine, cysteine, methionine, homocysteine, betaine, phenylalanine, tyrosine, tryptophan, proline, hydroxyproline, succinic acid, fumaric acid, citric acid, oxalic acid, formic acid, tartaric acid, α-hydroxy-*n*-caproic acid, α-hydroxy-*n*-butyric acid, α-hydroxy-isovaleric acid,

α-ketoglutaric acid, lactic acid, oxalacetic acid, sodium pyruvate (each tested at a concentration of 100 μg/ml), folic acid, citrovorum factor, ascorbic acid, thiamine, riboflavin, pyridoxal, biotin, nicotinic acid, *p*-aminobenzoic acid, $B_{12}$, choline chloride, calcium pantothenate, uracil, thymine, cytosine, adenine, guanine, xanthine, hypoxanthine, adenosine, guanosine, xanthosine, inosine, adenylic acid, and guanylic acid (each tested at a concentration of 10 μg/ml).

## Results

*Cross-resistance studies.* Two strains, one resistant to azaserine and the other resistant to *p*-fluorophenylalanine, were obtained from *E. coli* ATCC 9637 by serial transfer in increasing concentrations of the individual inhibitors. These cultures were found to be cross-resistant to both azaserine and *p*-fluorophenylalanine (Table 1).

TABLE 1. *Inhibitory activity of azaserine and p-fluorophenylalanine against Escherichia coli ATCC 9637 and derived resistant strains*

| Organism | Inhibitor to which strain derived as resistant | Minimal inhibitory concentration (μg/ml) determined by spread-plate procedure | |
|---|---|---|---|
| | | Azaserine | *p*-Fluorophenylalanine |
| *E. coli* ATCC 9637 | None | 0.075 | 3.0 |
| *E. coli*/AZA | Azaserine | >1000.0 | >1000.0 |
| *E. coli*/FPA | *p*-Fluorophenylalanine | >1000.0 | >1000.0 |

*Inhibition reversal studies in E. coli B96.* In preliminary studies the purine requirement of this bacterium was confirmed by use of the spread-plate technique. It was found that hypoxanthine or adenine concentrations as low as 30 μg/ml of medium were adequate for total recovery of inoculum organisms. Xanthine or guanine at 30 μg/ml of medium also supported growth of this organism, but incubation periods up to 72 hr were required for colony development. The corresponding ribonucleosides or ribonucleotides behaved in a manner identical to the free bases. No other metabolites or metabolite mixtures supported the growth of this culture in the absence of purine material.

The effect of metabolites on azaserine inhibition of *E. coli* B96 growing on medium supplemented with different purine materials was determined. When grown on medium containing 30 μg/ml of hypoxanthine, inosine, adenine, xanthine, xanthosine, guanine, guanosine, or guanylic acid, the minimal inhibitory concentration of azaserine was 0.003 to 0.01 μg/ml. Increasing the concentration of the purine substrate or adding mixtures of purine materials to the medium did not effect the minimal inhibitory concentration of azaserine for this organism. Phenylalanine, tyrosine, or tryptophan, individually tested

at concentrations of 100 μg/ml, reversed approximately 100 times the minimal inhibitory concentration of azaserine regardless of which purine was satisfying the growth requirement of the organism. All other individual metabolites tested failed to reverse azaserine action under these conditions. It has previously been shown in wild-type *E. coli* that, in the presence of small amounts of phenylalanine, histidine reverses azaserine. This same phenomenon was found to occur in *E. coli* B96 when cultured on adenine-supplemented medium (Fig. 1).

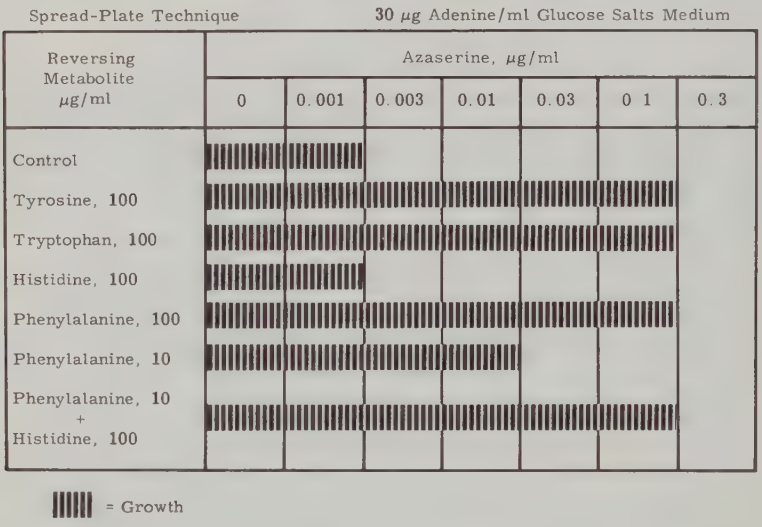


FIG. 1. *Effect of certain metabolites on the inhibition of Escherichia coli B96 by azaserine.*

Inhibition of *E. coli* B96 by *p*-fluorophenylalanine was reversed not only by phenylalanine, tyrosine, and tryptophan, but also by histidine, glutamine, and purines (Fig. 2). It may be noted that *p*-fluorophenylalanine, like azaserine, inhibited *E. coli* B96 at lower concentrations than were required to inhibit the wild-type *E. coli* (compare Fig. 2 with Table 1).

*Inhibition-reversal studies in E. coli M83-5.* Inhibition of the phenylalanine-requiring *E. coli* M83-5 by *p*-fluorophenylalanine was demonstrated. Such inhibition was reversed not only by phenylalanine but also by purines. Histidine was also a potent reversing agent (Fig. 3).

*Histidine and the growth-requirements of E. coli B96.* It was observed that histidine in concentrations as low as 10 μg/ml stimulated the growth of *E. coli* B96 in glucose-salts medium, containing

growth-limiting concentrations of purine. No other amino acid exerted a similar effect. Figure 4 illustrates this effect when *E. coli* B96 is grown in inosinic acid-supplemented liquid medium.

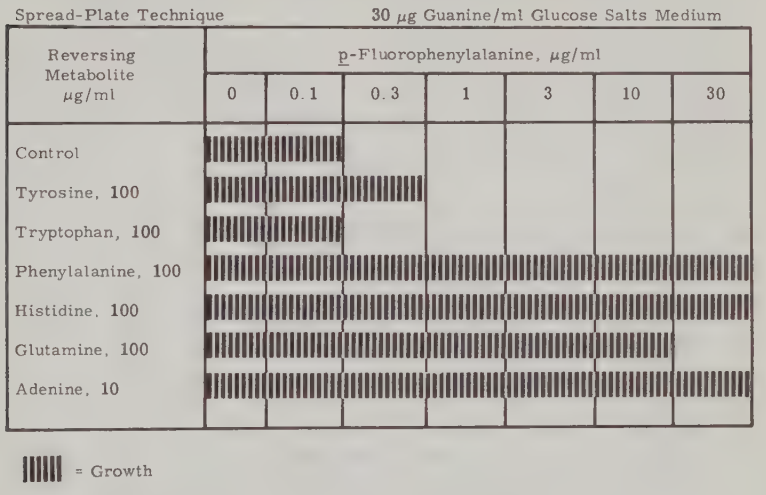


FIG. 2. *Effect of certain metabolites on the inhibition of Escherichia coli B96 by p-fluorophenylalanine.*

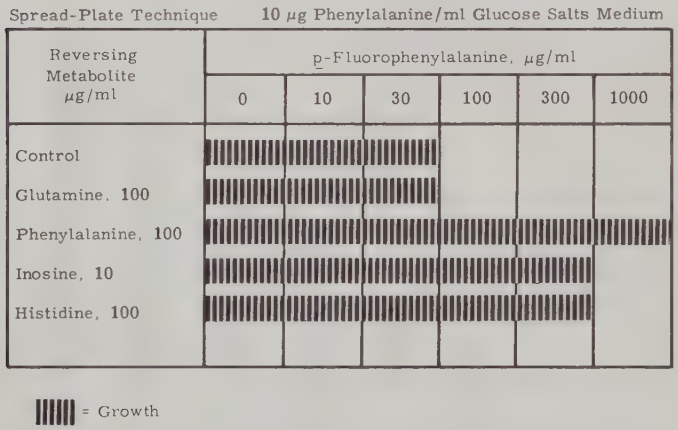


FIG. 3. *Effect of certain metabolites on the inhibition of Escherichia coli M83-5 (phenylalanine-requiring) by p-flurorphenylalanine.*

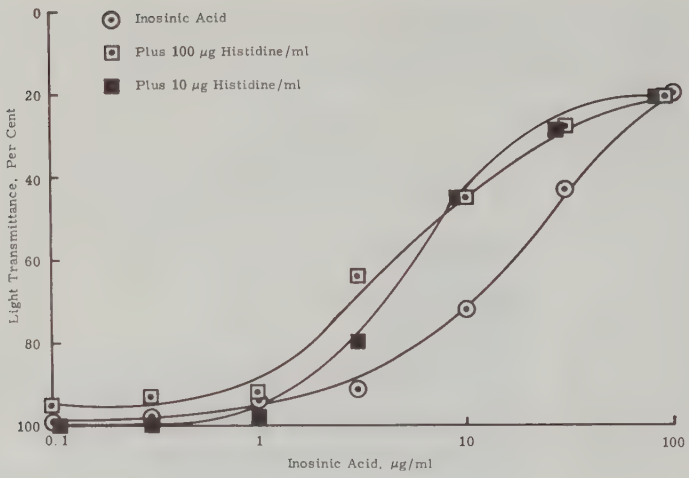


FIG. 4. *Effect of histidine on the purine requirement of Escherichia coli B96.*

*Histidine and the growth-requirements of E. coli M83-5.* In liquid medium histidine decreased the phenylalanine requirement of *E. coli* M83-5. Purines did not influence this phenylalanine requirement (Fig. 5).

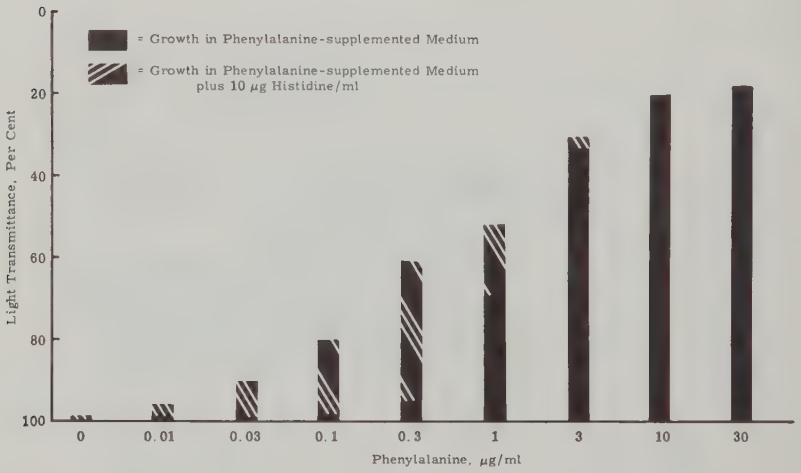


FIG. 5. *Effect of histidine on the growth response of Escherichia coli M83-5 to phenylalanine (liquid medium, 22 hr incubation).*

## Discussion

The work reported here was stimulated by the possibility that the first, most sensitive reaction(s) in *E. coli* inhibited by azaserine are not in the currently defined areas of purine synthesis or that phenylalanine plays a heretofore unrecognized role in purine metabolism. It would appear that both premises may be correct. If the prime site of azaserine attack in *E. coli* is in *de novo* purine biosynthesis at the formylglycinamide ribotide → formylglycinamidine ribotide reaction, than a bacterium lacking the ability to synthesize purines could be more resistant to azaserine action than the wild-type *E. coli*. Such a bacterium exists in *E. coli* B96, a mutant strain of *E. coli* B which lacks the enzymatic capacity to convert 4-amino-5-imidazolecarboxamide ribotide to inosinic acid. However, this organism proved to be more sensitive to azaserine than its parent. This increased sensitivity of *E. coli* B96 indicates inhibition of a growth-essential reaction other than formylglycinamide ribotide → formylglycinamidine ribotide and suggests that this reaction may also be a site of azaserine attack in the parent *E. coli*.

In view of the fact that the conversion of shikimic acid-5-phosphate to anthranilic acid in *E. coli* is azaserine-sensitive, the reversal of azaserine action in *E. coli* B96 by aromatic amino acids could be superficially explained on the basis of aromatic amino acids being the products of an azaserine-inhibited reaction. However, the fact that key intermediates, such as anthranilic acid, fail to reverse azaserine inhibition of growing cultures, while histidine and purines do reverse, would seem to negate this explanation. Furthermore, the concentrations of azaserine required to inhibit the phenylalanine-requiring strain of *E. coli* in the presence of limiting concentrations of phenylalanine would suggest that the shikimic-anthranilic acid conversion is less sensitive to azaserine than are other glutamine-requiring reactions. Thus, it appears that inhibition of aromatic amino acid synthesis is not a primary site of azaserine attack in *E. coli*.

In the phenylalanine-requiring strain of *E. coli*, not only phenylalanine but also histidine and purines reverse the inhibition caused by *p*-fluorophenylalanine, a reversal pattern very similar to that of azaserine in this same organism. Such similarities, coupled with the observed cross-resistance existing between resistant cultures obtained by passage through azaserine or *p*-fluorophenylalanine, can best be explained in terms of a common site (or sites) of action of these two inhibitors.

Over the past few years, considerable evidence has been obtained indicating that the biosynthesis of histidine begins with adenosine triphosphate (ATP). Moyed and Magasanik (1960) described the enzymatic formation of imidazoleglycerol phosphate, a histidine precursor, in a system containing a bacterial extract, ribose 5-phosphate, an

ATP-generating system, glutamine, and $Mg^{++}$. They demonstrated that APT was converted to 5-amino-1-ribosyl-4-imidazolecarboxamide-5'-phosphate, with the transfer of the purine N-1 and C-2 atoms to the ring of imidazoleglycerol phosphate. In the absence of glutamine this system accumulated an intermediate of uncertain structure (compound III) which appeared to be a bound form of 5-amino-1-ribosyl-4-imidazolecarboxamide-5'-phosphate. Recently it has been shown (Ames, Martin, and Garry, 1961; Klopotowski, Luzzati, and Slonimski, 1960) that *N*-1(5'-phosphoriboxyl)-ATP is a precursor of compound III and can be converted to it.

We suggest that a site of attack of azaserine may well be in this area and that phenylalanine may play an essential role in this reaction. Since it has been clearly demonstrated that phenylalanine reverses azaserine inhibition of the enzymatic conversion of formylglycinamide ribotide → formylglycinamidine ribotide, it is possible that phenylalanine may play an essential but undefined role in more than one enzymatic reaction. Such a function of phenylalanine could perhaps explain the broad activity of this amino acid in reversing inhibition of bacterial growth caused by a number of diverse types of inhibitors.

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## Literature Cited

Abrams, R., and M. Bentley. 1959. Biosynthesis of nucleic acid purines. III. Guanosine 5'-phosphate formation from xanthosine 5'-phosphate and L-glutamine. Arch. Biochem. Biophys. **79**:91-110.

Ames, B. N., R. G. Martin. and B. J. Garry. 1961. The first step of histidine biosynthesis. J. Biol. Chem. **236**:2019-2026.

Anderson, E. H. 1946. Growth requirements of virus-resistant mutants of *E. coli* strain B. Proc. Natl. Acad. Sci. U. S. **32**:120-128.

Bennett, L. L., Jr., F. M. Schabel, Jr., and H. E. Skipper. 1956. Studies on the mode of action of azaserine. Arch. Biochem. Biophys. **64**:423-436.

Buchanan, J. N., B. Levenberg, I. Melnick, and S. C. Hartman. 1957. The specific action of azaserine on enzymes concerned with purine biosynthesis, p. 523-540. *In* J. W. Rebuck, F. H. Bethell, and R. W. Monto, [ ed. ], The leukemias: etiology, pathophysiology, and treatment. Academic Press, Inc., New York.

Foster, J. W., and R. F. Pittillo. 1953. Reversal by complex natural materials of growth inhibition caused by antibiotics. J. Bacteriol **65**:361-367.

Gots, J. S., and S. H. Love. 1954. Purine metabolism in bacteria. II. Factors influencing the biosynthesis of 4-amino-5-imidazolecarboxamide by *E. coli*. J. Biol. Chem. **210**:395-405.

Hartman, S. C., B. Levenberg, and J. M. Buchanan. 1955. Involvement of ATP, 5-phosphoribosylpyrophosphate and L-azaserine in the enzymatic formation of glycinamide ribotide intermediates in inosinic acid biosynthesis. J. Am. Chem. Soc. **77**:501-503.

Kaplan, L., H. C. Reilly, and C. C. Stock. 1959. Action of azaserine on *Escherichia coli*. J. Bacteriol **78**:511-519.

Klopotowski, T., M. Luzzati, and P. P. Slonimski. 1960. Evidence for a new step between ATP and AICA ribotide in the cyclic process of histidine biosynthesis. Biochem. Biophys. Research Communs. **3**:150-154.

Moyed, H. S., and B. Magasanik. 1960. The biosynthesis of the imidazole ring of histidine. J. Biol. Chem. **235**:149-153.

Pittillo, R. F. 1961. Studies on the antimicrobial nature of action of azaserine, p. 276-287. *In* P. Gray, B. Tabenkin, and S. G. Bradley, [ ed. ], Antimicrobial agents annual. 1960. Plenum Press, New York.

Preiss, J., and P. Handler. 1958. Biosynthesis of diphosphopyridine nucleotide. II. Enzymatic aspects. J. Biol. Chem. **233**:493-500.

Reilly, H. C. 1954. The effect of amino acids upon the antimicrobial activity of azaserine. Proc. Am. Assoc. Cancer Research **1**:40.

Srinivasan, P. R. 1959. The enzymatic synthesis of anthranilic acid from shikimic acid-5-phosphate and L-glutamine. J. Am. Chem. Soc. **81**:1772-1773.

Tomisek, A. J., H. J. Kelly, and H. E. Skipper. 1956. Chromatographic studies of purine metabolism. I. The effect of azaserine on purine biosynthesis in E. coli using various $C^{14}$-labeled precursors. Arch. Biochem. Biophys. **64**:437-455.

Tomisek, A. J., M. R. Reid, and H. E. Skipper. 1959. Chromatographic studies of purine metabolism. IV. Reversal of azaserine-induced inhibition by phenylalanine and tryptophan. Cancer Research **19**:489-493.

# EFFECT OF STREPTOMYCIN ON THE BIOSYNTHESIS OF ERGOSTEROL IN GROWING CULTURES AND CELL-FREE PREPARATIONS OF YEASTS

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When examining possible pathways of biosynthesis of sterols from acetate, it was noted that one of the hypothetical intermediates bore an interesting resemblance to a compound which Umbreit (1953) had implicated in the mode of action of streptomycin. Umbreit's observations suggested that the prime function of streptomycin was to prevent the metabolism of this compound, 2-phospho-4-hydroxy-4-carboxy adipic acid. If this new phosphorylated compound or some compound similar to it were involved in the biosynthesis of sterols as predicted on the basis of an hypothesis, then the addition of streptomycin to a sterol-synthesizing system should inhibit that synthesis. The data presented in this paper show that sterol biosynthesis can be inhibited by streptomycin.

## Materials and Methods

*Saccharomyces carlsbergensis* (ATCC 9080) was grown in 200 ml of medium I (Klein, 1955) in 500-ml Erlenmeyer flasks, 4 flasks per treatment, and inoculated from a log phase culture on the same medium. Incubation was at 27 C with continuous shaking for 48 hr. Cells were harvested by centrifugation, washed twice with cold water, and suspended in water. Samples were taken for determination of dry weight and cholesterol as described by Klein (1955).

Cell-free preparations of bakers' yeast (Fleischmann's dry active yeast) were prepared by the technique developed by Alexander (1957). Desalting was accomplished with a column (4 × 10 cm) of Sephadex G-25. The preparation was used immediately (fresh) or frozen at -15 C. The frozen preparations were thawed and used after 1 to 5 days of storage. The preparation was incubated with appropriate additives in 50-ml beakers for 3 hr in air on a Dubinoff shaker at

27 C. The "complete" reaction mixture contained 4.0 ml enzyme (18 to 25 mg total protein); tris(hydroxymethyl)aminomethane·HCl (pH 7.0), 20.0 mg base; adenosine triphosphate (ATP), 1.0 mg; diphosphopyridine nucleotide (DPN), 1.0 mg; $MgSO_4 \cdot 7H_2O$, 8.0 mg; $K_2HPO_4$, 4.0 mg; coenzyme A (CoA), 0.5 mg; methionine, 2.5 mg; and labeled substrate containing 1 $\mu$c radioactivity. Where indicated, neutralized oxalacetic acid (OAA), 1.32 mg; hexose diphosphate (HDP), 4.5 mg; and freshly prepared streptomycin sulfate, 740 $\mu$g base/mg salt, were added. The final volume of the reaction mixture was 7.0 ml.

The order and time sequence of additions to the reaction mixture can alter the final results. In the experiments with cell-free preparations, all reagents except enzyme and substrate were mixed together. Enzyme was then added and followed immediately by subtrate.

The reaction was stopped by the addition of 25 ml of 1.0 M alcoholic KOH. After the further addition of 1 mg of purified ergosterol, 0.5 ml 0.1% hydroquinone, and a boiling stone, the mixtures were digested in a steam cone (ca. 3 hr) until the volume was reduced to 3 ml. Then, 3 ml of 95% (v/v) ethanol were added, and the solution transferred to a 50-ml glass-stoppered tube. The digestion beaker was rinsed with a total of 2 ml 40% ethanol (v/v) and the rinse combined with the digest. The digest was extracted twice with 8-ml and 4-ml portions of diethyl ether. The combined ether extracts were washed twice with 3-ml volumes of water and the wet ether extract evaporated to 0.3 ml on a 50 C water bath. Digitonides, prepared and purified by the method described by Vahouny et al. (1960), were collected directly on filter paper or on a thin layer of Celite supported by filter paper and dried over silica gel. The dry digitonides were counted in a windowless flow counter to the 0.05 level of significance. Radioactivity is reported as total net counts per minute per reaction mixture.

## Results and Discussion

Growing cultures of *S. carlsbergensis* were studied initially. Streptomycin sulfate, at concentrations of 0.1% or higher, inhibited growth in the Klein medium completely. At concentrations less than 0.1%, streptomycin had little effect upon growth, as measured by dry weight of cells produced. However, the ergosterol content of these cells was altered by sublethal concentrations of streptomycin (Table 1). There was an almost linear decrease in the amount of ergosterol produced with increasing concentrations of streptomycin.

The necessity for using high concentrations of streptomycin to alter growth and sterol production in the yeast was attributed to the impermeability of the intact organism to the added antibiotic (Berkman et al., 1948). To avoid this difficulty, cell-free preparations of yeast, capable of synthesizing sterol, were studied. The preparation

described by Alexander (1957) was selected because the desalting step in the procedure would remove extraneous salts which might prevent or reverse complex formations between the streptomycin and its site(s) of attachment (Donovick et al., 1948).

TABLE 1. *Effect of streptomycin on ergosterol formation in growing cultures of Saccharomyces carlsbergensis*

| Streptomycin-$SO_4$ concentration | Total growth in 200 ml medium | Ergosterol |
|---|---|---|
| % | *gm dry wt* | *mg/gm dry cells* |
| 0 | 0.332 | 10.7 |
| 0.025 | 0.319 | 9.3 |
| 0.050 | 0.322 | 7.9 |
| 0.075 | 0.317 | 5.1 |

Table 2 shows the cofactor requirements of the cell-free system. Maximal incorporation of acetate-$C^{14}$ into sterols was obtained with supplementation. Both the fresh and frozen preparations required ATP, DPN, CoA, methionine, and magnesium. The ortho phosphate requirement was not tested. Addition of OAA and HDP to the system had little effect on the fresh preparation but did stimulate the frozen one. Either OAA or HDP was stimulatory to the frozen preparation.

TABLE 2. *Cofactor requirements for incorporation of acetate-1-$C^{14}$ into sterols by cell-free system*

| Cofactor | Incorporation* | |
|---|---|---|
| | Fresh enzyme | Frozen enzyme |
| | *count/min* | *count/min* |
| None | 357 | 105 |
| Complete system | 2,580 | 747 |
| OAA + HDP added | 2,510 | 2,215 |
| OAA added | 2,600 | 2,050 |
| HDP added | 2,490 | 2,127 |
| ATP omitted | 1,675 | 580 |
| DPN omitted | 2,260 | 675 |
| CoA omitted | 2,432 | 718 |
| Methionine omitted | 1,760 | 557 |
| $Mg^{++}$ omitted | 1,840 | 637 |

*Total radioactivity in digitonides.

The effect of streptomycin upon the incorporation of acetate-1-$C^{14}$ into sterols is shown in Table 3. Fresh and frozen preparations were studied with and without OAA and HDP supplementation. Streptomycin inhibited the biosynthesis of sterol under all conditions, with fresh, frozen, supplemented, and unsupplemented preparations. Inhibition

was greater in frozen preparations than in fresh and in supplemented than in unsupplemented preparations. The ability to incorporate acetate into sterols varied from preparation to preparation, making quantitative comparison between different preparations of enzyme impossible. Similarly, the inhibition by streptomycin varied from preparation to preparation. It is clear, however, that streptomycin inhibited sterol synthesis under the conditions described.

TABLE 3. *Effect of streptomycin on incorporation of acetate-1-$C^{14}$ into sterols by cell-free preparations*

| Streptomycin·$SO_4$ | OAA and HDP | Incorporation* | |
|---|---|---|---|
| | | Fresh enzyme | Frozen enzyme |
| *μg/ml* | | *count/min* | *count/min* |
| 0 | 0 | 3,220 | 1,302 |
| 60.6 | 0 | 2,850 (11.5)† | 856 (33.0) |
| 152.0 | 0 | 2,530 (21.5) | 660 (49.3) |
| 0 | + | 2,980 | 3,420 |
| 60.6 | + | 2,470 (17.0) | 2,680 (21.6) |
| 152.0 | + | 2,046 (31.2) | 1,465 (57.2) |

*Total radioactivity in digitonides.
†Figures in parentheses indicate percentage inhibition.

The incorporation of acetate, mevalonate, and pyruvate into sterols and also the effect of streptomycin on the incorporation of these several substrates are shown in Table 4. The preparation used was fresh and supplemented with OAA and HDP. More radioactivity from the methyl carbon of acetate was incorporated into sterol than from the carboxyl group. The ratio of methyl carbon to carboxyl carbon incorporated is 1.82. When acetate is incorporated into cholesterol by liver, a ratio of 1.25 is obtained (Little and Bloch, 1950). Incorporation of a 2-labeled mevalonate was less than either type of acetate.

TABLE 4. *Effect of streptomycin on incorporation of various substrates into sterols by cell-free preparations*

| Substrate* | Streptomycin·$SO_4$ | Incorporation† |
|---|---|---|
| | *μg/ml* | *count/min* |
| Acetate-1-$C^{14}$ | - | 1,756 |
| Acetate-1-$C^{14}$ | 150 | 346 (80.4)‡ |
| Acetate-2-$C^{14}$ | - | 3,194 |
| Acetate-2-$C^{14}$ | 150 | 1,752 (45.0) |
| *d,l,* Mevalonate-2-$C^{14}$ | - | 633 |
| *d,l,* Mevalonate-2-$C^{14}$ | 150 | 253 (60.0) |
| Pyruvate-2-$C^{14}$ | - | 2,230 |
| Pyruvate-2-$C^{14}$ | 150 | 1,050 (51.9) |

*Each reaction mixture contained 1 μc of activity.
†Total radioactivity in digitonides.
‡Figures in parenthesis indicate percentage inhibition.

Slight incorporation of mevalonate was observed with two different batches of the acid from different sources, and it appears to be real for the system as constituted. Probable deficiency of manganese in the system could have caused low mevalonate incorporation because Tchen (1958) showed that mevalonic kinase required that ion for maximal activity. Although magnesium could substitute, it was not as effective as manganese. Incorporation of carboxyl-labeled acetate was inhibited more than that of the methyl label.

Wright and Hansen (1960) showed that the addition of protamine sulfate inhibited the biosynthesis of cholesterol in a liver preparation. This inhibition, which was quite striking in their system, was reversible with added ATP. The effect of protamine on a fresh preparation of yeast, supplemented with OAA and HDP, is shown in Table 5. Protamine was inhibitory to sterol synthesis at both levels of ATP shown. Protamine inhibition in the yeast system was not reversed by added ATP.

TABLE 5. *Effect of protamine on incorporation of acetate-1-$C^{14}$ into sterol by cell-free preparations*

| Protamine·$SO_4$ | ATP | Incorporation* |
|---|---|---|
| *mg* | *mg* | *count/min* |
| 0 | 0.2 | 5,740 |
| 5 | 0.2 | 4,360 (22.1)† |
| 10 | 0.2 | 2,390 (58.3) |
| 0 | 0.5 | 3,218 |
| 5 | 0.5 | 1,838 (42.8) |
| 10 | 0.5 | 1,258 (70.8) |

*Total radioactivity in digitonides.
†Figures in parenthesis indicate percentage inhibition.

Table 6 shows the effect of two levels of ATP upon streptomycin inhibition. Streptomycin (60 μg/ml) inhibited acetate incorporation 20% in the presence of 0.2 mg ATP and 8.2% in the presence of 0.5 mg ATP. Although extra ATP was itself inhibitory to incorporation, it did decrease the net effect of the streptomycin.

TABLE 6. *Effect of ATP concentration on streptomycin inhibition of acetate-1-$C^{14}$ incorporation into sterols*

| ATP | Streptomycine·$SO_4$ | Incorporation* |
|---|---|---|
| *mg* | *μg/ml* | |
| 0.2 | 0 | 5,270 |
| 0.2 | 60 | 4,215 (20.0)† |
| 0.5 | 0 | 3,162 |
| 0.5 | 60 | 2,900 ( 8.2) |

*Total radioactivity in digitonides.
†Figures in parenthesis indicate percentage inhibition.

Both streptomycin and protamine are inhibitory to sterol synthesis in the same concentration range. For example, at 10 mg protamine sulfate per reaction mixture, assuming a molecular weight of 3,000, the concentration was 0.48 $\mu$M/ml, and streptomycin sulfate at 152 $\mu$g/ml represented a concentration of 0.17 $\mu$M/ml. The inhibitory action of streptomycin in this concentration range probably cannot be attributed to precipitation of nucleic acid or nucleoprotein since Cohen and Lichtenstein (1960) showed that streptomycin at concentrations of 1/5 $\mu$M/ml precipitated only 10% of the deoxyribonucleic acid and none of the ribonucleic acid from *Escherichia coli* extracts.

The observation, that streptomycin inhibition was reversed by added ATP and the protamine effect was not, can indicate different binding energies of the protamine and streptomycin complexes, rather than different sites of action.

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## Literature Cited

Alexander, G. J. 1957. Preparation of cell-free yeast homogenate that converts acetate to sterols. Science **126**:1065.

Berkman, S., R. J. Henry, R. D. Housewright, and J. Henry. 1948. Streptomycin. IV. Absorption of streptomycin by susceptible and resistant bacteria. Proc. Soc. Exptl. Biol. Med. **68**:65-70.

Cohen, S. S., and J. Lichtenstein. 1960. The isolation of deoxyribonucleic acid from bacterial extracts by precipitation with streptomycin. J. Biol. Chem. **235**:PC 55-56.

Donovick, R., A. B. Bayan, P. Canales, and F. Pansy. 1948. The influence of certain substances on the activity of streptomycin. III. Differential effects of various electrolytes on the action of streptomycin. J. Bacteriol. **56**:125-137.

Klein, H. P. 1955. Synthesis of lipids in resting cells of *Saccharomyces cerevisiae*. J. Bacteriol. **69**:620-627.

Little, H. N., and K. Bloch. 1950. Studies on the utilization of acetic acid for the biological synthesis of cholesterol. J. Biol. Chem. **183**:33-46.

Tchen, T. T. 1958. Mevalonic kinase: Purification and properties. J. Biol. Chem. **233**:1100-1103.

Umbreit, W. W. 1953. The action of streptomycin. VI. A new metabolic intermediate. J. Bacteriol. **66**:74-81.

Vahouny, G. V., C. R. Borja, R. M. Mayer, and C. R. Treadwell. 1960. A rapid, quantitative determination of total and free cholesterol with anthrone reagent. Anal. Biochem. **1**:371-381.

Wright, L. D., and R. Hanson. 1960. Protamine and conversion of mevalonic acid to cholesterol by liver homogenates. Proc. Soc. Exptl. Biol. Med. **103**:854-857.

# ADAPTABILITY OF *NEUROSPORA* TO 6-DIAZO-5-OXO-L-NORLEUCINE AND RELATED INHIBITING AGENTS

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A new comprehensive list of *Neurospora crassa* stock cultures has become available (Barratt and Ogata, 1961). A careful study of this list and other similar reviews in literature (Beadle and Tatum, 1945; Houlahan, Beadle, and Calhoun, 1949; Barratt et al., 1954) confirmed the apparent fact that only three major types of mutants have been reported in *Neurospora*. These mutants are either biochemical mutants, those that require growth factors added to minimal medium, perhaps amino acids, purine-pyrimidines, vitamins, or other organic specific substances; morphological mutants which differ in color or growth pattern from the wild type; or strains that accumulate products or intermediate metabolites of their chemical pathways in the medium.

Resistant mutants to normally chemical-inhibiting substances have not been reported for *Neurospora*; furthermore, this mutant type has not been observed among thousands of cultures treated with chemical inhibitors (Fuerst, 1960*a*). Resistant mutants are common in bacteria against streptomycin (Goldstein, Magasanik, and Umbarger, 1955), atabrine (Sevag and Gots, 1948), and numerous other antimetabolites, as well as in mammalian systems, when tumor cells exposed to antineoplastic agents become drug-resistant (Rhoads, 1955).

The investigation presented here consists of attempts to adapt *N. crassa* to gradually increasing inhibitory doses of antimetabolites to determine whether or not it is possible to establish chemical-resistant strains in this organism.

## Materials and Methods

*History of strains.* The test organism used in this investigation was the pink bread mold, *N. crassa*. The specific wild-type strains employed were Em5256A, KJTa, and Pa8005A. Em5256A was obtained from Florida State University; the KJTa strain came from the University of Texas; and Pa8005A from Stanford University. These strains were kept in stock on minimal medium agar slants.

*Growth media and supplements.* Except where otherwise stated, growth of *Neurospora* was measured as dry mycelial weight after 72 hr of incubation at 25 C in 25 ml of the usual Fries medium 3 (minimal) in 125-ml Erlenmeyer flasks. A drop of the inoculum, consisting of a spore suspension of microconidia in sterile water, was applied to each flask with a 1-ml pipette. The supplements added to the minimal medium in some of the experiments were mixtures of amino acids, vitamins, purines and pyrimidines, organic acids, and yeast extract. The complete medium, used in some of the tests with the flask method, consisted of Difco yeast extract, 2.5 g; malt extract, 2.5 g; Bacto peptone, 2.0 g; casein hydrolysate, acid, 2.0 ml; glycerol, 20.0 ml; and minimal medium minus sucrose, 1,000 ml.

The medium used for the sexual crossings of the two mating types of *Neurospora* was Difco corn meal agar, 1.7 g corn meal agar per 1,000 ml of water.

*Procedure of adaptation.* The plate-well method of Fuerst and Skellenger (1958) has been slightly modified to be used for the adaptation problem. The petri plates containing minimal medium and 4% agar were perforated with a sterile cork borer, and four or five wells were made in the medium. Into the wells, varying doses of the inhibitor were pipetted with a micropipette, and a drop of *Neurospora* conidial suspension was added. The growth around the wells was measured and recorded in millimeters. Hyphae growing out of the wells were inoculated into plates containing higher concentrations than the plates from which the inocula were obtained. Subsequently, the adapted strains were transferred for storage on agar slants containing the inhibitor.

*Spore-shooting method.* The spore-shooting method of Strickland (1960) was slightly modified and employed in experiments in which the mature ascospore would shoot to a petri plate lid containing 4% agar. A block of this agar, containing the ascospore, was then removed with a dissecting needle under the microscope and transferred into a small tube of either minimal or complete liquid medium. Sometimes spores were not picked but allowed to germinate after heat activation by floating the lid in a 60 C water bath for half an hour. Varying concentrations of antimetabolites were incorporated into the 4% agar minimal medium in the petri plate lids to which the spores were shot. A list of some of the antimetabolites used in the adaptation studies is shown in Table 1.

## Results

Results of using the plate-well method to adapt *N. crassa* Em5256A to some inhibitors are shown in Table 1. Selection of the inhibitors to be used was based on some earlier work by Fuerst (1960*b*).

TABLE 1. *Adaptation of Neurospora crassa Em5256A to inhibitory chemicals, using the plate-well method*

| Inhibitor | Initial | | Final | | Total number of consecutive transfers | Code number for adapted strains of Em5256A* |
|---|---|---|---|---|---|---|
| | Inhibiting concentration | Mycelia growth | Tolerated concentration | Mycelia growth | | |
| | *mg* | *mm* | *mg* | *mm* | | |
| *Successful adaptations:* | | | | | | |
| 4-Aminopyrazolo-(3,4-D) pyrimidine | 0.24 | 5 | 4.06 | 5 | 17 | (4-APP-1) |
| 8-Azaguanine | 0.35 | 0 | 0.61 | 9 | 2 | (8-azag.-1) |
| Adenine | 0.34 | 0 | 2.21 | 25 | 14 | (adenine-α) |
| 2,6-Diaminopurine sulfate | 1.17 | 3 | 17.51 | 25 | 6 | |
| 6-Diazo-5-oxo-L-norleucine | 0.0017 | 0 | 0.0004 | 4 | 3 | (DON-1) |
| 6-Diazo-5-oxo-L-norleucine | 0.0017 | 0 | 0.0043 | 34 | 8 | (DON-α) |
| Methotrexate | 0.15 | 0 | 2.40 | 13 | 4 | |
| DL-Thioctic acid | 0.83 | 0 | 0.62 | 26 | 3 | (thioc.-1) |
| *Unsuccessful adaptations:* | | | | | | |
| 2-Aminopyrimidine salt of nitros malonitrite | 0.24 | 0 | 0.14 | 0 | 2 | |
| | | | 0.19 | 0 | 2 | |
| Azaserine | 0.30 | 0 | 0.10 | 0 | 2 | |
| | | | 0.24 | 0 | 2 | |
| | | | 0.30 | 0 | 2 | |
| 4-Heptylamino-1-methylpyrazolo(3,4-D) pyrimidine hydrochloride | 0.14 | 0 | 0.07 | 0 | 2 | |
| 1-Methyl-4-octyl-aminopyrazolo-(3,4-D) pyrimidine | 0.35 | 0 | 0.28 | 0 | 2 | |
| 4-(2-Thienyl) butanoic acid | 0.18 | 0 | 0.10 | 0 | 2 | |
| | | | 0.15 | 0 | 2 | |
| | | | 0.20 | 0 | 2 | |

*Use of Greek α in code number, instead of Arabic numeral, indicates that the strain was adapted within the last four months of the investigation, without prolonged refrigeration prior to testing.

It should be noted that 6-diazo-5-oxo-L-norleucine (DON) was the most effective inhibitor to which Em5256A could be adapted. The adaptation of this strain to many times the initial fully inhibitory dose of adenine, 4-amino-pyrazolo(3,4-*d*)pyrimidine (4-APP), azaguanine, 2,6-diaminopurine sulfate, methotrexate, aminopterin, and DL-thioctic acid was also successful. There were some chemical agents to which *Neurospora* could not be adapted (Table 1). Strains adapted and further investigated are shown in Table 2.

TABLE 2. *Neurospora crassa Em5256A adaptations to antimetabolites that were further investigated*

| Antimetabolite | Initial fully inhibitory dose/well | Adapted concentration per well | Treatment of strain after adaptation (stored on 3 ml of 2% agar minimal medium) | | Code numbers of adapted strains of Em5256A* |
|---|---|---|---|---|---|
| | | | Plus | Kept | |
| | *mg* | *mg* | | | |
| Adenine | 0.34 | 2.21 | 4 ml of 4 mM adenine | at 28-30C | (adenine-α) |
| 4-Amino-pyrazolo (3,4-d) pyrimidine | 0.24 | 4.06 | 0.4 ml of 300 mM 4-APP | at 2C for 2 yr and at 28-30C for 6 months | (4-APP-1) |
| 8-Azaguanine | 0.35 | 0.61 | 80 μl of 50 mM 8-azaguanine | at 2C for 2 yr and at 28-30C for 6 months | (8-azag.-1) |
| 6-Diazo-5-oxo-L-norleucine | 0.002 | 0.003 | 0.1 ml of 0.25 mM DON | at 2C for 2 yr | (DON-1) |
| 6-Diazo-5-oxo-L-norleucine | 0.002 | 0.004 | 1.0 ml of 0.25 mM DON | at 28-30C | (DON-α) |
| 6-Diazo-5-oxo-L-norleucine | 0.002 | 0.0003 | 0.1 ml of 0.25 mM DON | at 2C for 2 yr and at 28-30C for 6 months | (DON-3) |
| DL-Thioctic acid | 0.83 | 0.62 | 15 μl of 200 mM DL-thioctic acid | at 2C for 2 yr and at 28-30C for 6 months | (thioc.-1) |

*Use of Greek α in code number, instead of Arabic numeral, indicates that the strain was adapted within the last four months of the investigation, without prolonged refrigeration prior to testing.

*N. crassa* KJTa has also been successfully adapted to several antimetabolites (Table 3). The code numbers for the adapted strains of KJTa to DON, DL-thioctic acid, and adenine are KJTa(DON-α), KJTa(thioc.-α), and KJTa(adenine-α), respectively.

No data of extensive studies on the storage of the adapted cultures are given here. However, these investigations showed that essentially the keeping of the cultures in the refrigerator gave results that were similar, whether the organisms had been stored for 1 month or 2 years, even though the 2-year storage required several transfers of the stock cultures. Testing of the adapted strains with the flask method proved to be disappointing. Coenocytic conditions, analogous to those of heterocaryosis, obscured the expression of the adapted nuclei. In consequence of the inability to prove successful adaptation with the flask method, it seemed necessary to make sexual crosses between the adapted strains with wild types and to study the progenies.

TABLE 3. *Progeny of cross number 1, Em5256A(DON-3) × KJTa, as compared to the progeny of cross number 2, Em5256A × KJTa*

| Germination medium | Total spores picked | Spores | | Growth pattern | |
|---|---|---|---|---|---|
| | | Not germinated | Germinated | Wild-type | Fragmented |
| | *no.* | *no.* | *no.* | *no.* | *no.* |
| *complete* | | | | | |
| cross 1 | 100 | 17 | 83 | 18 | 65 |
| cross 2 | 100 | 42 | 58 | 58 | 0 |
| *minimal* | | | | | |
| cross 1 | 100 | 83 | 17 | 17 | 0 |
| cross 2 | 100 | 48 | 52 | 52 | 0 |

The data illustrated in Table 3 show that the progenies from the DON-adapted strain to the unadapted strain resulted in three types of offspring: those that would germinate apparently normally, those that were definitely lethal, and the so-called fragments which may be considered semilethal. Fragments were obtained from ascospores that germinated and sent out a short hyphal tube which then failed to grow further. Fragments were obtained only when the spore germinated on complete medium, in which case two-thirds of all offspring were of this general semilethal variety (Table 3). On minimal medium no fragments were encountered. Fragments were also observed from a cross of Em5256A(thioc.-1) × KJTa. Short-time exposure of the wild-type Em5256A to inhibitory doses of thioctic acid would also produce 8% of fragments in the progenies as compared to 35% for the long-time adapted strains.

Attempts to grow fragments on any supplement tested were unsuccessful. The addition of DON to the medium of the DON-adapted strains does not enhance the growth of the fragments, and an elevation in temperature does not induce growth.

Experiments were set up in order to find out what substance(s) in complete medium permits the semilethal fragmentation strains to germinate. L-Norleucine and adenine did not prove effective in overcoming DON inhibition. Other individual constituents of complete medium have not yet been investigated in detail for relief of DON toxicity, because of failure to stimulate sufficient growth in the fragments.

In experiments with shooting ascospores of strains adapted to DON, evidence is given in favor of the presence of DON-resistant nuclei in the progenies from DON-adapted parent(s) (Table 4). When both strains of the cross have been adapted to DON, a higher ratio of DON-resistant nuclei can be isolated than when one or no strain of the cross has been adapted to DON. Table 4 also indicates that when the wild-type strain has been freshly adapted to the inhibitor

TABLE 4. *Isolation of DON-resistant progeny from ascospores shot to plates of minimal medium containing DON*

| Maternal strain of Em5256A | Paternal strain of KJTa | Progenies of crosses* | | | | |
|---|---|---|---|---|---|---|
| | | 0.0 ml | 0.1 ml | 0.5 ml | 1.0 ml | 1.5 ml |
| | | *no.* | *no.* | *no.* | *no.* | *no.* |
| wild-type | wild-type | OG‡ | 7 | 0 | 0 | 0 |
| (-DON-3)† | wild-type | OG | 25 | 6 | 1 | 0 |
| (-DON-α) | wild-type | OG | 31 | 13 | 4 | 0 |
| (-DON-3) | (-DON-α) | OG | 21 | 8 | 14 | 0 |
| (-DON-α) | (-DON-α) | OG | OG | 5 | 3 | 2 |

*Crosses germinated after 48 hr on 45 ml of 4% agar-minimal medium containing DON (in ml of 2.5 mM DON per plate).

†Parentheses indicates adaptation to DON.

‡OG = overgrown.

and not stored in the refrigerator, the ratio of isolation of resistant nuclei from a cross with KJTa is higher than when Em5256A is adapted to DON and stored. Quantitative data are presented as evidence that indeed, from all strains exposed to DON and of all strains adapted for resistance to this compound, it is possible to isolate resistant offspring. The control was most sensitive to the inhibitor, whereas from both adapted strains crossed to each other, a lower sensitivity was obvious. Table 4 also shows that in all of these crosses, the maternal strain used was Em5256A. This is owing to difficulties in obtaining perithecia formation from other types of crosses. In general, from all crosses where KJTa adapted to DON has been used as the maternal parent, no perithecia formation resulted (Table 5). The normal cross, with KJTa as the maternal parent, always gave perithecia.

TABLE 5. *Infertile crosses on corn meal agar*

| Strains | |
|---|---|
| Maternal | Paternal |
| Em5256A(DON-1) | KJTa |
| Em5256A(DON-1) | KJTa(DON-α) |
| KJTa(DON-α) | Em5256A |
| KJTa(DON-α) | Em5256A(DON-α) |
| KJTa(DON-α) | Em5256A(DON-1) |
| KJTa(DON-α) | Em5256A(DON-3) |

Data, similar to that from DON-resistant progenies of the crosses shown previously, have been obtained from a cross between Em5256A (thioc.-1) with KJTa. Unfortunately, experiments so far are preliminary, because the exact range of most effective differences, analogous to Table 4, has not yet been determined. The trend, however, indicates that drug-resistant nuclei can also be obtained from this cross.

## Discussion

The perplexing problem of why *N. crassa* does not normally induce chemical-resistant mutants has been investigated, and the data indicate that it is possible to obtain such mutants. However, methods had to be applied that would favor the detection of such drug-resistant strains. The multinucleate condition in *Neurospora* mycelium seems to obscure the expression of nuclei that were able to survive increasing amounts of DON and other substances tested. When the incidence of semilethals, referred to as fragments, was noticeably high, survival of such nuclei apparently depended on products produced by the normal nuclei with which they were associated in the vegetative stage. Storage of the resistant strains is possible, but does present additional problems.

Crossing the adapted strains to wild type resulted in isolation of progeny from the treated nuclei, thus permitting expression of chromosomal damage produced as the result of exposure to the inhibitors. Similarly, resistance to DON and other agents was demonstrated.

Actually, if one examines methods used on bacteria and compares them to the effective tests applied on *Neurospora,* one must admit that perhaps the method used determines whether or not the existence of drug-resistant strains can be observed. With other methods the genetic balance of *Neurospora* does not favor the expression of such mutants.

The methods used to find nuclei and to grow those that are chemical resistant in *Neurospora* are actually very close to the methods used in bacterial analyses. Perhaps it indicates that one can detect such mutants most easily if methods for the detection are favorable and selective, and least easily when one wishes to force the organisms into a standard pattern of analysis which up to then has not yielded resistant mutants.

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## Literature Cited

Barratt, R. W., D. Newmeyer, D. D. Perkins, and L. Garnjobst. 1954. Map construction in *Neurospora crassa*, p. 1-93. *In* M. Demerec [ed.], Advances in genetics, vol. 6. Academic Press, New York.

Barratt, R. W., and W. N. Ogata. 1961. *Neurospora* stock list. Compiled at Fungal Genetics Stock Center, Botany Department, Dartmouth College, Hanover, N. H.

Beadle, G. W., and E. L. Tatum. 1945. Methods of producing and detecting mutations concerned with nutritional requirements. Am. J. Botany **32**:678-685.

Feurst, R. 1960*a*. Screening of antineoplastic agents with *Neurospora crassa*, p. 101-107. *In* Developments in Industrial Microbiology, vol. 1, Plenum Press, New York.

Feurst, R. 1960*b*. Variations in *Neurospora crassa*, p. 211-220. *In* Developments in Industrial Microbiology, vol. 1, Plenum Press, New York.

Fuerst, R., and W. M. Skellenger. 1958. A *Neurospora* plate method for testing antimetobolites. Antibiotics & Chemotherapy **8**:76-80.

Goldstein, A., B. Magasanik, and H. E. Umbarger. 1955. On the mechanism of development of resistance to streptomycin in *Micrococcus pyogenes* var. *aureus*. J. Bacteriol. **70**:620-621.

Houlahan, M. B., G. W. Beadle, and G. Calhoun. 1949. Linkage studies with biochemical mutants of *Neurospora crassa*. Genetics **34**:493-507.

Rhoads, C. P., [ed.]. 1955. Antimetabolites and cancer: a symposium. Ann. N. Y. Acad. Sci. p. 89-105.

Sevag, M. G., and J. S. Gots. 1948. Enzymatic studies on the mechanism of the resistance of *Pneumococcus* to drugs. J. Bacteriol. **56**:709-748.

Strickland, W. N. 1960. A rapid method for obtaining unordered *Neurospora* tetrads. J. Gen. Microbiol. **22**:583-588.

# BIOGENESIS OF THE NEOMYCINS

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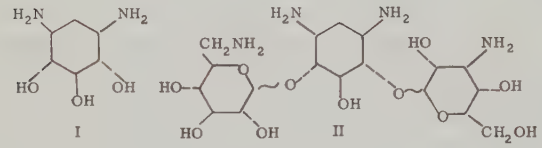
The neomycins (Waksman and Lechevalier, 1949) are the oldest members of the family of antibiotics characterized by the presence of a central deoxystreptamine nucleus (I). Other well-characterized individuals in the family include kanamycin B (II, Cron et al., 1958*a*) and paromomycin (III, Haskell, French, and Bartz, 1959), which now has been shown (Schillings and Schaffner, 1962) to be identical to catenulin, hydroxymycin, and aminosidin and is at least very closely related to zygomycin A.



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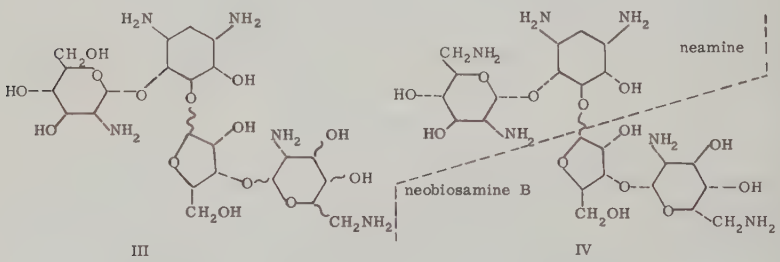



III IV

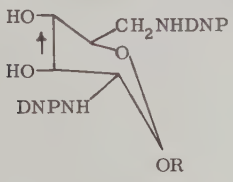
In the neomycin branch of the family, individual members (in particular, neomycins B and C) have been identified. These isomers both contain the same neamine moiety, which accounts for approximately half the molecule, but contain isomeric neosamines B and C in the neobiosamine (B and C) moieties. The major component of the usual neomycin complex is neomycin B; it is now possible to assign a structure (IV) to this molecule.

The structure of neamine was established recently (Carter et al., 1961). The structure of neobiosamine B was assigned earlier, except for stereochemistry of neosamine B at C-1, C-3, C-4, and C-5 (Rinehart et al., 1960).

## Experimental

In this study methyl neobiosaminide B was oxidized with sodium periodate. The crude product was treated with 2,4-dinitrofluorobenzene and hydrolyzed, to give a product (L-isoserinaldehyde-DNP) of strongly negative rotation. Similar treatment of 6-amino-6-deoxy-D-glucose (Cron et al., 1958*b*) gave a corresponding product (D-isoserinaldehyde-DNP) of strongly positive rotation. Thus C-5 in neosamine B has the L configuration, opposite to that in neosamine C (2,6-diamino-2,6-dideoxy-D-glucose). The stereochemistry of C-3 and C-4 follows from the observation that *N,N'-bis*-(dinitrophenyl)-neosamine B and the corresponding dinitrophenyl derivatives of neobiosamine B and methyl neobiosaminide B are not oxidized at all by sodium periodate at 30 C. Although it could be argued that this lack of reactivity is due simply to the adjacent bulky dinitrophenyl group's shielding of the C-3 hydroxyl group from periodate complex formation, this argument is invalidated by rapid periodate uptake by the corresponding dinitrophenyl derivatives of neosamine C and methyl neobiosaminide C. Therefore, the unreactivity of the neosamine B-DNP derivatives is best ascribed to an unusual conformation of neosamine B in these derivatives, a conformation which prevents the formation of the periodate complex required for cleavage.

In neosamine C derivatives, the diaminohexose undoubtedly assumes the C-1 conformation (V, Reeves, 1949), in which the two vicinal hydroxyl groups (and, indeed, all substituents except the α C-1 alkoxy group in neobiosamine C and methyl neobiosaminide C) are equatorial and subtended by an approximately 60° dihedral angle (α).

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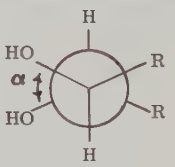
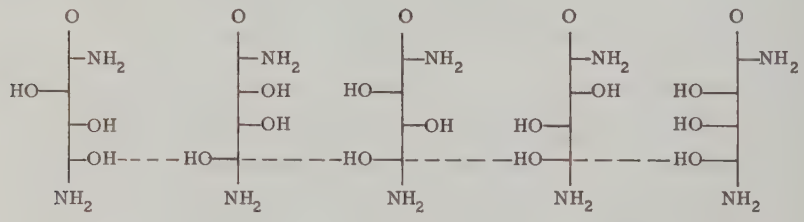


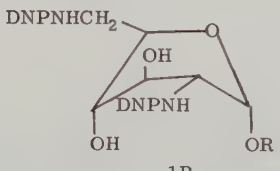

For neosamine B is is required, to explain the torpidity of its DNP derivatives toward periodate, that the two hydroxyl groups at C-3 and C-4 be *trans* with respect to one another. Otherwise, the

maximal dihedral angle subtended, with the pyranose ring in either a chair or a boat form, is 60°, as in neosamine C derivatives. Thus, L-altrose and L-talose configurations are eliminated, leaving L-idose and L-mannose as the two possibilities. Conformational considerations do not allow a choice between these two configurations, but that of L-idose is preferred for neosamine B, since this configuration differs from that of neosamine C (D-glucose stereochemistry) only at the C-5 carbon atom, while L-mannose stereochemistry would differ at C-3 and C-4 as well.



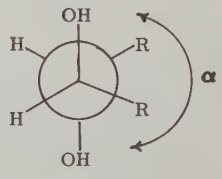

The isolation from the same source of sugars epimeric at a single carbon atom is well known. For instance, D-glucosamine and D-galactosamine (C-4 epimers) occur in the closely related hyaluronic acid and chondroitin sulfate (Meyer, 1957), and D-glucuronic acid is found in chondroitin sulfates A and C and L-iduronic acid (epimeric at C-5) in chondroitin sulfate B (Hoffman, Linker, and Meyer, 1956).

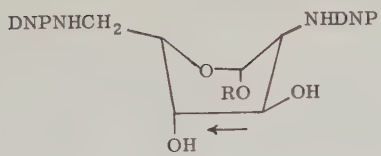
Although a definite conformation can not be assigned to L-idose-diamine derivatives, it may be noted that the C-1 conformation requires the bulky $-CH_2NHDNP$ group at C-5 (and a ribose moiety at C-1) to be axial, while the C-1 conformation introduces axial substituents at C-2, C-3, and C-4. It is possible for the DNP-substituted groups at C-2 and C-5 both to be equatorial only in a boat or a modified boat form, as in VI (1B conformation) and VII (B2). In the former conformer, the C-3 and C-4 hydroxyl groups are separated by a dihedral angle $\alpha = 180^{\circ}$; in the latter, by $\alpha = 120^{\circ}$.



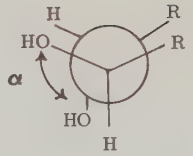

1B

VI






B2
VII



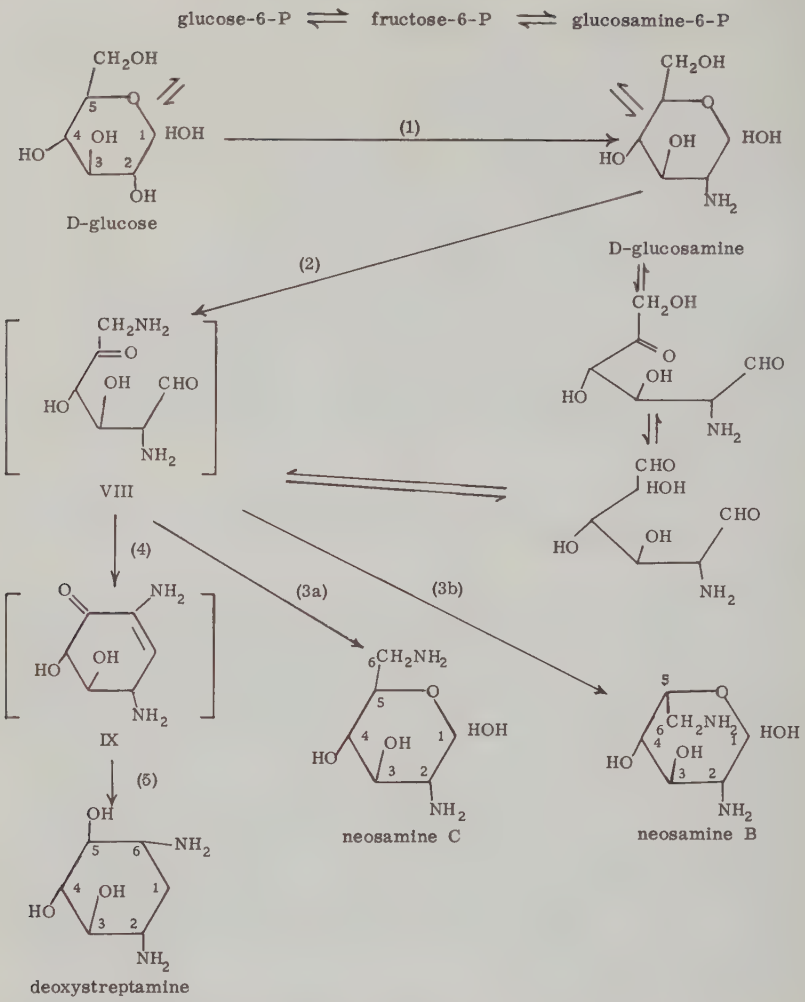

The linkage between neosamine B and D-ribose may be assigned the $\alpha$ configuration, from use of Hudson's rules, as applied in the similar assignment of an $\alpha$ linkage to neobiosamine C (Rinehart, Woo, and Argoudelis, 1958). It is not yet known with certainty whether D-ribose exists in the furanose or pyranose form in the intact neomycin, but furanose is tentatively assigned.[1] The ribose ring size, the $\alpha$ or $\beta$ linkage of ribose to deoxystreptamine, and the absolute stereochemistry of the 4-substituted deoxystreptamine are the remaining structural points.

The similarity in stereochemistry of the neosamine B, neosamine C, and deoxystreptamine moieties of neomycin B invites consideration of their biogenetic pathway. The general scheme below is suggested.

In support of this over-all scheme, uniformly labeled D-glucose has been shown to be easily incorporated into neomycin, though no degradation of the intact labeled molecule was attempted (Sebek, 1955). Within this scheme, step (1), the intact conversion of D-glucose-$1C^{14}$ to D-glucosamine-$1\text{-}C^{14}$ is well established (Becker and Day, 1953). The conversion of D-glucosamine to the intermediate 5-keto-2,6-diaminohexose (step 2) appears to have no precedent. However, the intermediate 5-keto- and 6-aldo-aminohexoses find analogy in that a 5-keto-6-deoxyaldohexose is a portion of the antibiotic hygromycin (Mann and Woolf, 1957), which also contains neoinosamine, a 2-amino-2-deoxymyoinositol (Allen, 1956), and more recently a 6-aldo-ketohexose has been isolated (Weidenhagen and Bernsee, 1960). Amination of the aldo- or keto-hexosamine by the glucose → glucosamine pathway would give the 2,6-diamino-5-keto-aldohexose (VIII) shown.

The key intermediate VIII could then either be reduced (perhaps by DPNH) to 2,6-diamino-D-glucose (step 3a) and 2,6-diamino-L-idose (step 3b), or undergo aldol cyclization (step 4) to give the unsaturated cyclohexenone IX. Conversion of IX to deoxystreptamine (step 5) requires only that the two-mole reduction give at both asymmetric centers the more stable (equatorial) isomer. It may be noted that nearly equal amounts of neosamine B and neosamine C are apparently produced by *Streptomyces fradiae*, with neosamine C (the more stable) slightly predominating. Neosamine C is found in neamine in both neomycins and in the neobiosamine C moiety of neomycin C, but neosamine B is found only in neomycin B, the predominant isomer.

[1] Note added in proof: furanose form now confirmed by O-methylation.




In the proposed biogenetic route, the neosamines formed are probably then joined to D-ribose and deoxystreptamine. It is also possible, however, that the glycosidic linkages are established before cyclization of VIII to give deoxystreptamine. In that case, the absolute configuration of substituted deoxystreptamines would follow directly, since no glycosidically 5-substituted D-glucose derivatives are known, but 3-substituted compounds are well established (as in hyaluronic acid). Of course, the 4-glycosidic linkage found in substituted deoxystreptamines is common, as in cellulose, starch, and chitin.

## Literature Cited

Allen, G. R., Jr. 1956. The synthesis of certain degradation products of the antibiotic 1703-18B. The synthesis of *neo*-inosamine-2. J. Am. Chem. Soc. **78**:5691-5692.

Becker, C. E., and H. G. Day. 1953. Utilization of glucosone and the synthesis of glucosamine in the rat. J. Biol. Chem. **201**:795-801.

Carter, H. E., J. R. Dyer, P. D. Shaw, K. L. Rinehart, Jr., and M. Hichens. 1961. The structure of neamine. J. Am. Chem. Soc. **83**:3723-3724.

Cron, M. J., O. B. Fardig, D. L. Johnson, D. F. Whitehead, I. R. Hooper, and R. U. Lemieux. 1958a. Kanamycin. IV. The structure of kanamycin. J. Am. Chem. Soc. **80**:4115.

Cron, M. J., O. B. Fardig, D. L. Johnson, H. Schmitz, D. F. Whitehead, I. R. Hooper, and R. U. Lemieux. 1958b. Kanamycin. II. The hexosamine Units. J. Am. Chem. Soc. **80**:2342.

Haskell, T. H., J. C. French, and Q. R. Bartz. 1959. Paromomycin. IV. Structural studies. J. Am. Chem. Soc. **81**:3482-3483.

Hoffman, P., A. Linker, and K. Meyer. 1956. Uronic acid of chondroitin sulfate B. Science **124**: 1252.

Mann, R. L., and D. O. Woolf. 1957. Hygromycin. III. Structure studies. J. Am. Chem. Soc. **79**:120.

Meyer, K. 1957. The chemistry of the mesodermal ground substances. Harvey Lectures 1955-1956, p. 88.

Reeves, R. E. 1949. Cuprammonium-glycoside complexes. III. The conformation of the D-glucopyranoside ring in solution. J. Am. Chem. Soc. **71**:215-217.

Rinehart, K. L., Jr., A. D. Argoudelis, T. P. Culbertson, W. S. Chilton, and K. Striegler. 1960. Chemistry of the neomycins. VI. Structure of neobiosamine B. J. Am. Chem. Soc. **82**:2970-2971.

Rinehart, K. L., Jr., P. W. K. Woo, and A. D. Argoudelis. 1958. Chemistry of the neomycins. Isolation of neosamines B and C. Stereochemistry of neobiosamine C. J. Am. Chem. Soc. **80**:6461-6462.

Schillings, R. T., and C. P. Schaffner. 1962. Differentiation of catenulin-neomycin antibiotics; Identity of catenulin, paromomycin, hydroxymycin, and aminosidin. Antimicrobial Agents and Chemotherapy - 1961, p. 274-285.

Sebek, O. K. 1955. The synthesis of neomycin-$C^{14}$ by *Streptomyces fradiae*. Arch. Biochem. Biophys. **57**:71-79.

Waksman, S. A., and H. A. Lechevalier. 1949. Neomycin, a new antibiotic active against streptomycin-resistant bacteria, including tuberculosis organisms. Science **109**:305-307.

Weidenhagen, R., and G. Bernsee. 1960. A new crystalline dicarbonyl sugar (6-aldo-D-fructose). Angew. Chem. **72**:109.

# DIFFERENTIATION OF CATENULIN-NEOMYCIN ANTIBIOTICS; IDENTITY OF CATENULIN, PAROMOMYCIN, HYDROXYMYCIN, AND AMINOSIDIN

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The neomycin complex (B and C; Swart, Hutchinson, and Waksman, 1949), catenulin (Davisson, Solomons, and Lees, 1952), and the more recently reported kanamycins (Takeuchi et al., 1957; Cron et al., 1958) are water-soluble, basic antibiotics which are markedly similar in chemical, physical, and biological properties. They can be readily differentiated, however, by paper chromatography of the respective sulfates of their *N*-acetylated derivatives (Rinehart et al., 1960). Paper chromatography of the three antibiotics has served not only for purposes of differentiation, but has also emphasized their chemical similarities, i.e., they are carbohydrate-like substances containing amino-sugar moieties. More detailed isolation and characterization of these components have established that the three antibiotics have certain identical sugar moieties in common.

As a consequence of the above evidence, these antibiotics may be classified as members of the general "neomycin" family, and may be considered apart from other water-soluble, basic antibiotic families of the streptomycetes.

Extensive antibiotic-screening programs throughout the world have frequently led to the isolation of the same antibiotic by different investigators and to its description under different names. Recently, this laboratory has established the identity of framycetin and neomycin B (Rinehart et al., 1960). It appeared from the literature, personal communications, and unpublished data from our laboratory, that the three antibiotics, paromomycin (Haskell, French, and Bartz, 1959), hydroxymycin (Hagemann, Nominè, and Pènasse, 1958), and aminosidin (Arcamone et al., 1959) were essentially similar in their chemical, physical, and biological properties to the earlier reported antibiotic, catenulin (Davisson et al., 1952.

The present study shows that when these four antibiotics are subjected simultaneously to various chemical and physical tests they are, indeed, indistinguishable and identical.

## Materials and Methods

*Antibiotics.* A sample of catenulin (lot #4080-74-C) was obtained from Walter Celmer of Chas. Pfizer and Co., Inc., Groton, Conn. Paromomycin is marketed by Parke, Davis and Co. under the name of Humatin. The paromomycin components, paromamine trihydrochloride (3446 x 15), methyl paromobiosaminide dihydrochloride (3552 x 47B), and paromose dihydrochloride (3285 x 57A) were obtained from T. H. Haskell of Parke, Davis and Co., Detroit, Mich. A sample of hydroxymycin (2654-1136) was obtained from M. G. Hagemann of U.C.L.A.F. - Sofrapen-Roussel, Paris, France. A sample of aminosidin was obtained from Farmitalia, Milan, Italy.

*Preparation of N-acetyl derivatives of the antibiotics.* The *N*-acetyl derivatives were prepared by a procedure similar to that reported in more detail in a previous paper (Rinehart et al., 1960). The antibiotic salts were converted to the free base by the use of the anion-exchange resin, Amberlite IRA 400 (hydroxyl phase). Each of the free bases was dissolved in absolute methyl alcohol; an excess of acetic anhydride was added, and the resultant solution was shaken at room temperature until the reaction mixture gave a negative ninhydrin test. The impure *N*-acetyl preparations were precipitated from solution by the addition of an excess of anhydrous ethyl ester. The precipitates were thoroughly washed with ethyl ether and dried in vacuo.

*Paper chromatography of N-acetyl derivatives.* The *N*-acetyl derivatives were chromatographed by the descending technique for 18 hr on Whatman no. 1 paper in a solvent system composed of *n*-butyl alcohol-pyridine-water (3:2:2, v/v). The *N*-acetylated antibiotics were detected on the paper chromatograms by a method reported for *N*-acetylated neomycin (Pan and Dutcher, 1956).

*Preparative cellulose-column chromatography.* The major component from each of these impure *N*-acetyl antibiotic preparations was isolated by a preparative chromatographic procedure similar to that reported in greater detail in a previous paper (Rinehart et al., 1960). The crude *N*-acetylated antibiotic sample was applied to a cellulose-powder partition chromatographic column and developed with the same solvent system used in the paper chromatography. The eluate was collected in samples of 2.0 ml. Paper chromatography and weight-residue analyses of appropriate fractions permitted the evaluation of the chromatographic fractionation and the location of major and minor components. The fractions containing the major components were pooled, extracted with petroleum ether, and concentrated to dryness in vacuo. The residues were dissolved in absolute methyl alcohol. Excess ethyl ether was added slowly with stirring. The *N*-acetyl derivatives in this case slowly precipitated in fine texture, due to the stirring. The precipitates were washed three times with small amounts of cold ethyl ether and dried in vacuo

over phosphorous pentoxide. The *N*-acetyl antibiotic was crystallized from aqueous acetone. The derivative was dissolved in a minimal amount of water, and acetone was added dropwise until a faint cloudiness persisted. The solution was placed in the refrigerator, whereupon crystals settled out. These crystals were washed with cold acetone and dried in vacuo over phosphorous pentoxide.

*Physical and analytical measurements.* Optical rotation determinations of the four *N*-acetylated antibiotics were made on 1.5% solutions in water at 23 C. The infrared spectra were obtained on a Baird-Atomic infrared spectrophotometer employing KBr tablets. The elemental and *N*-acetyl analyses were made by the Schwarzkopf Microanalytical Laboratory, Woodside, N.Y.

*Acid hydrolyses.* The *N*-acetyl antibiotic preparations were hydrolyzed, both with 6 N and 2 N hydrochloric acid, at a concentration of 5 mg of *N*-acetyl derivative per 0.25 ml of acid in sealed capillary tubes at 100 C for 1 hr. The antibiotic hydrochlorides were hydrolyzed in 6 N hydrochloric acid. The hydrolyzates were applied directly to Whatman no. 1 paper without prior removal of excess acid, and chromatographed by the descending technique in the solvent system *n*-butyl alcohol-pyridine-water-acetic acid (6:4:3:1, v/v) for 18 hr. The paper chromatograms of the hydrolyzates were prepared in duplicate and sprayed with a ninhydrin reagent composed of 0.25 g of ninhydrin in 100 ml of pyridine-water (1:1, v/v) and with an aniline acid phthalate reagent composed of 1.66 g of phthalic acid and 0.93 g of aniline in 100 ml of water-saturated *n*-butyl alcohol. Densitometer curves of the chromatograms were run on a Spinco Model R Analytrol recording densitometer.

For the preparative cellulose-powder partition chromatographic separation of the 6 N hydrochloric acid hydrolyzate of catenulin hydrochloride, the same solvent system was employed. Column fractions were analyzed with ninhydrin reagent, and appropriate fractions were pooled for recovery.

*Methanolysis.* Catenulin, paromomycin, hydroxymycin, aminosidin, and neomycin B were refluxed for 3 hr in 0.3 N anhydrous methanolic HCl at a concentration of 10 mg per ml. The resulting solutions were applied directly to Whatman no. 1 paper and developed for 18 hr with the same solvent system used for hydrolysis. Both ninhydrin and aniline acid phthalate reagents were employed in spraying the paper chromatograms.

*Periodate oxidation and hydrobromic acid hydrolysis.* Both *N*-acetyl catenulin and catenulin-free base were used in periodate-uptake studies. Since it was necessary to use small quantities (about 10 mg) of the antibiotics, the quantitative periodate oxidations were carried out by a modification of the spectrophotometric method reported by Aspinall (1957). The oxidations were carried out in the dark at 3 C for 15 hr. A fivefold excess of periodate (0.02 M sodium metaperiodate) was employed.

To determine the nature of the periodate uptake of catenulin and *N*-acetyl catenulin, the periodate reaction mixtures were first deionized by passage through a mixed-bed ion-exchange resin (Amberlite IRA 400 in the hydroxyl phase and Amberlite IRC 120 in the hydrogen phase). The eluate was concentrated to dryness in vacuo. One portion of the residue was hydrolyzed with 6 N HCl at 100 C for 1 hr, and another portion with 58% HBr at 100 C for 5 hr. After hydrolysis, the excess hydrochloric and hydrobromic acids were removed by repeated evaporations from aqueous solutions in vacuo. The resultant residues were analyzed by paper chromatography, with development in the same solvent system as above.

## Results and Discussion

The comparison and differentiation of neomycin-type antibiotics have always been difficult problems. The existence of complexes with varying composition has only contributed to the difficulty of the problem, and to the confusion and duplication of results found in the literature. The highly basic nature of the neomycins is a prime reason for the difficulty of their fractionation and characterization. Therefore, conversion to the neutral *N*-acetyl derivatives has been a useful technique in the differentiation of these antibiotics.

Pure *N*-acetyl derivatives of the neomycin-type antibiotics may be crystallized and are well suited for analytical characterization. Furthermore, paper chromatography and preparative cellulose-column partition chromatography of *N*-acetyl neomycin preparations revealed minor isomeric components which were not detected when the salts or free bases of these antibiotics were chromatographed. Therefore, the *N*-acetyl derivatives of the four antibiotics, catenulin, paromomycin, hydroxymycin, and aminosidin were prepared and subjected to both paper and preparative cellulose-column chromatography. Figure 1 shows a typical paper chromatogram obtained with these impure *N*-acetyl preparations, revealing three components in common. As expected, these preparations gave negative reactions with ninhydrin, both on paper and in solution. Further, reacetylation or passage through the cation-exchange resin, Amberlite IRC 50 ($H^+$), did not alter the paper chromatographic results (Fig. 1). It appears that, as has been shown in the case of the neomycin complex, these three spots may represent minor and major isomeric components. The relative amounts of each component in a particular preparation might tend to confuse the quantitative results obtained in in vitro antimicrobial spectral comparisons of the four antibiotics. In general, all four antibiotics seem to consist of one major antibiotic component in complex with varying amounts of two minor components. This may account in part for any differences in chemical, physical, and biological properties reported in the literature. In the paper

chromatography of the *N*-acetyl derivatives of the four commercial antibiotics, heavy concentrations of derivative had to be applied to the paper chromatogram to detect the minor components.

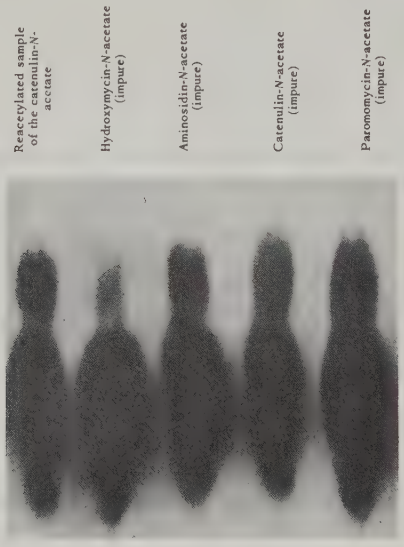


FIG. 1. *Paper chromatography of the N-acetyl derivatives of catenulin, paromomycin, hydroxymycin, and aminosidin complexes. Solvent system: n-butyl alcohol-pyridine-water (3:2:2, v/v).*

For comparison of the major components of the four *N*-acetylated antibiotics, preparative cellulose-powder partition column chromatography was employed in the separation of the *N*-acetylated complex. Weight-residue and paper-chromatographic analyses permitted the proper pooling of fractions. The major *N*-acetylated component was thus prepared in pure form after appropriate precipitation techniques.

The infrared spectrum for each of the pure major *N*-acetylated antibiotic preparations is given in Fig. 2. The striking similarity of all four preparations is most evident. Further analytical determinations, such as elemental analyses for carbon, hydrogen, and nitrogen, analyses for *N*-acetyl groups, and optical rotation, are reviewed in Table 1. These results also strongly suggest the identity of the major *N*-acetylated components of the four antibiotics.

Hagemann et al. (1958), after microanalysis of crystalline *N*-benzoyl hydroxymycins, proposed a molecular formula for hydroxymycin free base: $C_{25}H_{47}O_{15}N_5$. Arcamone et al. (1959) proposed for aminosidin free base a molecular formula of $C_{23}H_{45}O_{14}N_5$, based on microanalyses of both crystalline *N*-benzoyl and *N*-acetyl aminosidin.

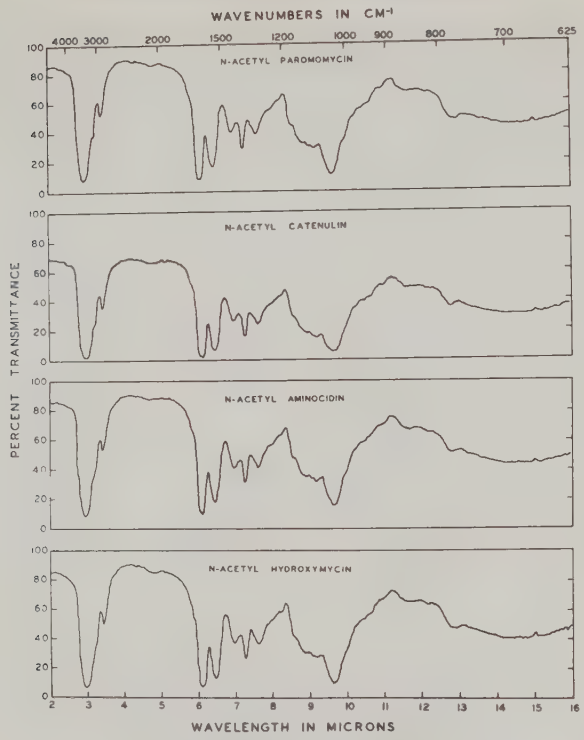


FIG. 2. *Infrared spectra of the N-acetyl derivatives of catenulin, paromomycin, and aminosidin (1% KBr).*

TABLE 1. *Comparison of the elemental and N-acetyl analyses and specific optical rotation*

| *N*-acetylated antibiotic | Carbon | Hydrogen | Nitrogen | *N*-acetyl | Specific rotation* |
|---|---|---|---|---|---|
| | % | % | % | % | |
| Paromomycin | 42.50 | 7.91 | 7.39 | 19.57 | + 63.0 |
| Catenulin | 42.50 | 7.23 | 7.33 | 19.22 | + 62.5 |
| Aminosidin | 42.66 | 6.69 | 7.51 | 19.52 | + 62.5 |
| Hydroxymycin | 42.14 | 7.09 | 7.39 | 19.08 | + 63.0 |

*$[\alpha]_D^{23}$ (c = 1.5 in water).

However, as shown in Table 2, the theoretical elemental analyses for the *N*-benzoyl derivatives of the two antibiotics, based on their different free base formulae, would be essentially the same. The experimental elemental analysis could support either of the two

formulae. Haskell et al. (1959), on the basis of elemental analysis of *N*-acetyl paromomycin and the results from degradation and characterization of the components of paromomycin, established a molecular formula for paromomycin free base as $C_{23}H_{45}O_{14}N_5$, which is the same as that proposed for aminosidin by Arcamone et al.

TABLE 2. *Elemental analyses of N-acyl derivatives*

| Reference | Derivative and its formula | Carbon | Hydrogen | Nitrogen |
|---|---|---|---|---|
| | | % | % | % |
| Hagemann et al. (1958) | *N*-benzoyl hydroxymycin ($C_{60}H_{67}N_5O_{20}$) | 61.20* 61.30 | 5.68 5.70 | 5.95 6.00 |
| Arcamone et al. (1959) | *N*-benzoyl Aminosidin ($C_{58}H_{65}N_5O_{19}$) | 61.3 – | 5.73 – | 6.17 – |
| Arcamone et al. (1959) | *N*-Acetyl aminosidin ($C_{33}H_{55}N_5O_{19}\cdot H_2O$) | 46.97 – | 6.81 – | 8.30 – |
| Haskell et al. (1959) | *N*-acetyl paromomycin ($C_{33}H_{55}N_5O_{19}\cdot H_2O$) | 46.97 46.64 | 6.81 7.25 | 8.30 8.17 |
| Schillings (1960) | *N*-acetyl hydroxymycin ($C_{33}H_{55}N_5O_{19}\cdot H_2O$) | 46.97 46.74 | 6.81 7.08 | 8.30 7.96 |
| Hagemann et al. (1958) | *N*-acetyl hydroxymycin ($C_{35}H_{57}N_5O_{20}\cdot H_2O$) | 47.41 – | 6.66 – | 7.92 – |

*The upper figure for each derivative represents the theoretical value; the lower figure is the experimental value.

After examining the analytical data reviewed in Table 2, it is quite clear that no differentiation of the antibiotics can be made. Included are elemental analytical data for a sample of the *N*-acetyl derivative of the hydroxymycin complex. These data are in better agreement with the other values cited in Table 2 than with those cited in Table 1 for the pure major *N*-acetylated components of the four antibiotics. Differences in the analytical results for the pure *N*-acetyl derivatives are due to hydration, since the carbon-nitrogen ratio is in agreement with the theoretical value (based on the formula proposed for paromomycin free base). The pure *N*-acetyl antibiotic components were found to be hygroscopic by nature.

The paper chromatographic studies, functional group and elemental analysis, optical rotation, and infrared spectral analyses all strongly suggest the identity of the major components of the catenulin, paromomycin, hydroxymycin, and aminosidin antibiotic complexes. The conclusion derived from these studies was further substantiated by comparative degradative studies.

Rinehart et al. (1960) showed by deamination procedures that direct acid hydrolysis of glycosidic bonds which are in proximity to amino groups is difficult, owing to inhibition by the positively charged amino group of the protonation required for cleavage of the glycosidic bond. In fact, easily destroyed molecules, such as pentoses, are degraded to furfural under acidic conditions severe enough to cause glycosidic cleavage. On the basis of this evidence, the neutral *N*-acetyl derivatives of the four antibiotics were hydrolyzed in both 2 N and 6 N HCl. Figure 3 shows a chromatogram of a 45-min hydrolyzate of the four impure *N*-acetyl derivatives in 6 N HCl. The densitometer curves of this chromatogram reveal a quantitative difference in some of the components, but all are qualitatively the same (Fig. 4). Since the antibiotics differ in the relative amounts of the minor and major components, this would be expected. When the *N*-acetyl derivatives were hydrolyzed in 2 N HCl, a reducing spot corresponding to D-ribose was found. This component was not found when 6 N HCl was employed for the hydrolysis. Acid hydrolysis of the pure *N*-acetyl antibiotic derivatives, followed by paper chromatographic analyses, produced results identical for all of the four antibiotics and not very unlike those results obtained with the complex antibiotic preparations.

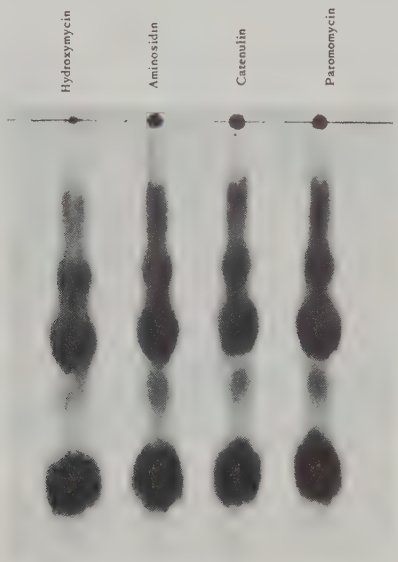


FIG. 3. *Paper chromatogram of the acid hydrolyzates of the N-acetyl derivatives of catenulin, paromomycin, hydroxymycin, and aminosidin. Solvent system: n-butyl alcohol-pyridine-water-acetic acid (6:4:3:1, v/v).*

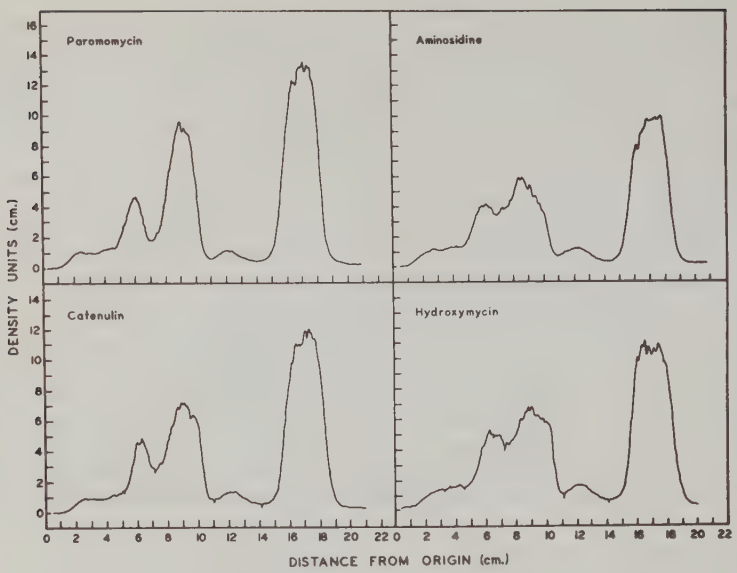


FIG. 4. *Densitometer curves from paper chromatography of the acid hydrolyzates of the N-acetyl derivatives of catenulin, paromomycin, hydroxymycin, and aminosidin. Solvent system: n-butyl alcohol-pyridine-water-acetic acid (6:4:3:1, v/v).*

The structure for paromomycin shown in Fig. 5 has been proposed (Haskell et al., 1959). A chromatogram of the 1-hr hydrolyzate of the hydrochlorides of paromomycin, catenulin, and hydroxymycin in 6 N HCl, with reference components derived from paromomycin, paromamine, 2-deoxystreptamine, and páromose, is illustrated

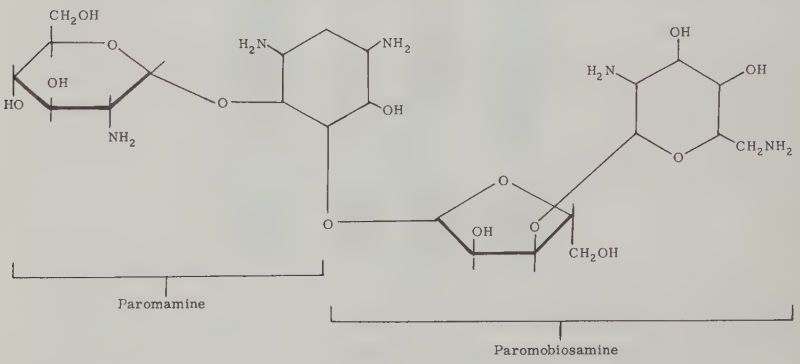


FIG. 5. *Proposed structural formula of paromomycin (Haskell et al., 1959).*

in Fig. 6. The component with the highest $R_F$ value corresponds to glucosamine. It was found that 2-deoxystreptamine is not released from paromamine, a complex of 2-deoxystreptamine and glucosamine, in detectable amounts until after 45 min. Paromamine in catenulin hydrolyzates was isolated by preparative paper chromatography and hydrolyzed. Paper chromatography of the hydrolyzates revealed components corresponding to 2-deoxystreptamine and glucosamine. Bartos (1958) isolated and characterized from hydroxymycin a product, which he called "pseudoneamine," similar to paromamine.

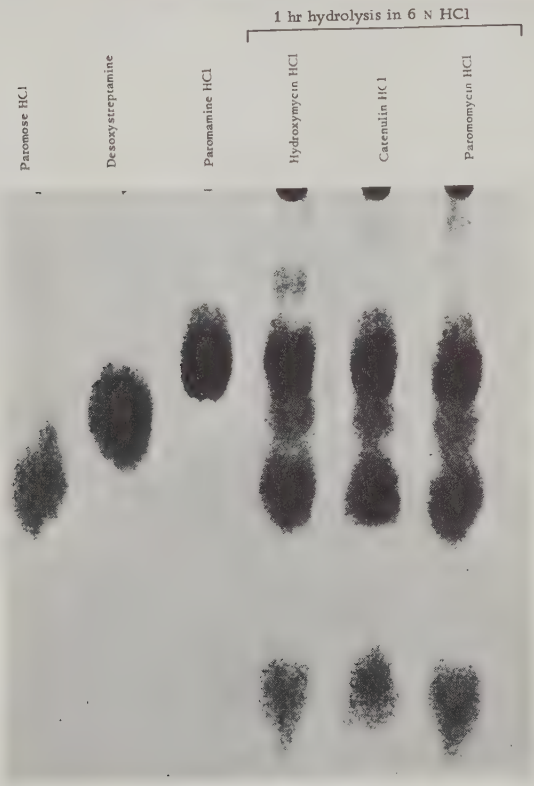


FIG. 6. *Paper chromatogram of the acid hydrolyzates of the hydrochlorides of paromomycin, catenulin, and hydroxymycin. Solvent system: n-butyl alcohol-pyridine-water-acetic acid (6:4:3:1, v/v).*

The methanolysis of neomycin B produces two fragments, neamine and methyl neobiosaminide B. Paromomycin was reported to yield methyl paromobiosaminide and paromamine on methanolysis.

Comparative methanolysis of catenulin, paromomycin, hydroxymycin, aminosidin, and neomycin B was followed by paper chromatography (Fig. 7). It is quite evident that catenulin, hydroxymycin, and aminosidin produce methanolysis products with similar $R_F$ values to those of the control samples, methyl paromobiosaminide and paromose (derived from the methanolysis of paromomycin). The reducing amino sugar, paromose, is produced by the further degradation of methyl paromobiosaminide. The nonreducing methanolysis component, paromamine, exhibits the lowest $R_F$ value. The identity of catenulin, paromomycin, hydroxymycin, and aminosidin and their nonidentity to neomycin B is further illustrated by these methanolysis studies.

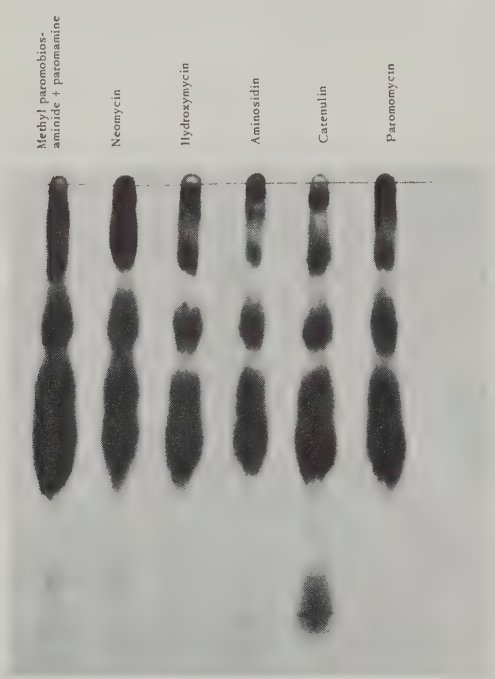


FIG. 7. *Paper chromatogram of methyl paromobiosaminide, paromamine, and the methanolysates of catenulin, paromomycin, hydroxymycin, aminosidin and neomycin B. Solvent system: n-butyl alcohol-pyridine-water-acetic acid (6:4:3:1, v/v).*

Haskell et al. (1959) reported that the quantitative uptake of periodate for *N*-acetyl paromomycin was 1.94 moles in 22 hr in 0.05 M periodate solution. On the assumption that catenulin and paromomycin have the same molecular weight, it was found that the periodate uptake for *N*-acetyl catenulin is 1.8 moles in 20 hr in 0.02 M periodate solution.

Considerations of the structural formula for paromomycin and the fact that periodate cleaves the bond between vicinal hydroxyl groups or vicinal hydroxyl and amino groups predict that, upon periodate oxidation of the free base of paromomycin, 2-deoxystreptamine will be destroyed. In the *N*-acetyl derivative, where there are no vicinal hydroxyl and amino groups, 2-deoxystreptamine will not be destroyed. After subjecting both catenulin free base and *N*-acetyl catenulin to periodate oxidation, followed by deionization of the oxidation mixtures by passage through a mixed bed of cationic- and anionic-exchange resins, the resulting products were heated with 48% hydrobromic acid on a steam bath for 5 hr. Hydrobromic acid destroys all reducing components, but does not destroy the nonreducing 2-deoxystreptamine. After removal of the excess hydrobromic acid, the resulting products were analyzed by paper chromatography. As predicted from the paromomycin formula, 2-deoxystreptamine was destroyed in the periodate oxidation of catenulin free base but not in the oxidation of *N*-acetyl catenulin, suggesting that in catenulin the 5,6 (4,5) hydroxyls of 2-deoxystreptamine are substituted.

## Literature Cited


Arcamone, F., C. Bertazzoli, M. Ghione, and T. Scotti. 1959. Aminosidina: un nuovo antibiotico oligosaccharidico. Giorn. microbiol. **7**:251-272.

Aspinall, G. O. 1957. Periodate oxidations by a spectrophotometric method. Chemistry & Industry, p. 1216-1217.

Bartos, M. J. 1958. Sur la << pseudo neamine >> produit d'hydrolyse de l'hydroxymycine. Ann. pharm. franc. **16**:596-600.

Cron, M. J., D. L. Evans, F. M. Palermiti, F. D. Whitehead, I. R. Hooper, P. Chu, and R. U. Lemieux. 1958. Kanamycin. V. The structure of kanosamine. J. Am. Chem. Soc. **80**:4741.

Davisson, J. W., I. A. Solomons, and T. M. Lees. 1952. Catenulin, a new antibiotic. Antibiotics & Chemotherapy **2**:460-461.

Hagemann, G., G. Nominè, and L. Pènasse. 1958. Sur un antibiotique, l'hydroxymycine produit par un Streptomyces. Ann. pharm. franc. **16**:585-596.

Haskell, T. H., J. C. French, and Q. R. Bartz. 1959. Paromomycin. IV. Structural studies. J. Am. Chem. Soc. **81**:3482.

Pan, S. C., and J. D. Dutcher. 1956. Separation of acetylated neomycins band C by paper chromatography. Anal. Chem. **28**:836-838.

Rinehart, K. L., A. D. Argoudelis, W. A. Goss, A. Sohler, and C. P. Schaffner. 1960. Chemistry of the neomycins. V. Differentiation of the neomycin complex. Identity of framycetin and neomycin B. Compounds from methyl neobiosaminide. J. Am. Chem. Soc. **82**:3938.

Schillings, R. T. 1960. Thesis, Rutgers University, New Brunswick, N. J.

Swart, E. A., D. Hutchison, and S. A. Waksman. 1949. Neomycin, recovery and purification. Arch. Biochem. **24**:92-103.

Takeuchi, T., T. Hikiji, K. Nitta, S. Yamazuki, S. Abe, H. Takayama, and H. Umezawa. 1957. Biological studies on kanamycin. J. Antibiotics (Japan) **10A**:107-114.

# COMPARISON OF TWO NEW TETRACYCLINES WITH TETRACYCLINE AND DEMETHYLCHLORTETRACYCLINE

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Tetracycline antibiotics have been used on a large scale during the past 15 years to treat a variety of infectious diseases. Dozens of analogues have been synthesized or produced by fermentation, with the hope that more effective compounds might be developed. The present study is concerned with an evaluation of antibacterial activity in vitro, and serum concentrations after oral administration in volunteers, of two new tetracycline compounds that show potential promise for clinical therapy.

## Materials and Methods

6-Methylene oxytetracycline (hereafter referred to as GS), known also as GS-2876, was supplied by Chas. Pfizer and Co. It was supplied in the form of laboratory powder in 20-mg ampules, and in 150-mg capsules of the hydrochloride. These capsules assayed at 152 mg per capsule (average of five capsules).

Ay 60042 (hereafter referred to as Ay) was originally synthesized by the Midy Co. in Milan, Italy; in Europe it is known as Mepicicline and as PZT. This tetracycline analogue is described as being highly soluble in water, and has been used extensively in Italy as an intramuscular dosage form in combination with phenoxymethylpenicillin, with the trade name Penetracyn. Chemically, a fairly long side chain has been added to the carbonyl amide of the tetracycline molecule, giving a larger molecule (mol wt 586.3) than the other tetracyclines. The chemical name is 4'-8-hydroxyethyl-diethylenediaminomethyl tetracycline, and the drug was supplied by Ayerst, McKenna, and Harrison (Montreal, Canada) as laboratory powder in 25-mg ampules, and in 150-mg capsules, assaying at 140 mg per capsule in terms of tetracycline.

Tetracycline (TC) was available as commercial hard-shell capsules containing 250 mg of tetracycline hydrochloride, with citric

acid filler (Lederle Achromycin V). Two different commercial lots were used, one with an assay value of 254 mg (lot no. 4859-222) and the other with a value of 268 mg (lot no. 4859-308). Demethylchlortetracycline (DMCT) consisted of soft-shelled capsules containing 150 mg of DMCT hydrochloride. Again two batches were used, one assaying at 162 mg (Lederle Declomycin lot no. 7-9868) and the other at 159 mg per capsule (Lederle Declomycin lot no. 9208-3009). Assays of all the above capsules were performed by the U.S. Food and Drug Administration through the courtesy of C. N. Lewis.

## Results

*Minimal inhibitory concentrations.* A twofold tube dilution test, using a 1:100 dilution of an 18-hr culture as the inoculum, was employed for testing the four antibiotics against representative clinical pathogens. GS and DMCT were roughly equal in activity, with GS being slightly more active against gram-positive bacteria, and DMCT being somewhat more active against the gram-negative bacteria. TC was intermediate, and Ay was the least active of the four on a weight basis (Table 1).

TABLE 1. *Susceptibility of recently isolated pathogens to four tetracycline antibiotics**

| Pathogens† | No. of strains | GS | DMCT | TC | Ay |
|---|---|---|---|---|---|
| Pneumococci | 6 | 0.05 | 0.07 | 0.13 | 0.17 |
| Group A streptococci | 6 | 0.06 | 0.06 | 0.15 | 0.18 |
| Susceptible staphylococci | 7 | 0.2 | 0.31 | 0.46 | 1.6 |
| Coliforms | 7 | 3.6 | 1.66 | 2.97 | 10.94 |

*The results are expressed as the minimal inhibitory concentrations (μg/ml).
†Each inoculum was a 1:100 dilution of an 18-hr culture.

*Serum binding.* The degree of serum binding of the four antibiotics was determined in two ways. In the first, the minimal inhibitory concentration of a group A streptococcus was determined in broth and in 50% serum with a tube dilution method, using small increments of antibiotic from one tube to the next. It was assumed that the tube giving inhibition in 50% serum contained the same amount of free antibiotic as was present in broth. For example, if the minimal inhibitory concentration was 0.03 μg/ml in broth, and growth was inhibited in 50% serum in the tube containing 0.3 μg/ml, 0.27 μg or 90% was considered to be bound by protein.

The second method consisted of ultrafiltration through a cellophane membrane, using air under pressure to allow sufficient antibiotic to pass through the membrane within 3 hr for assay by a

standard cup-plate method. The fluid containing 50% serum was adjusted to pH 6.9 prior to filtration.

There was remarkably good agreement with the two methods (Table 2). The amount of antibiotic bound was roughly the same for GS and DMCT, and was in excess of 80%. In contrast, less than 60% was bound for TC and Ay. The figures for binding are higher than some others published in the literature, due probably to differences in experimental methods, but it is felt that the values are valid from a comparative standpoint.

TABLE 2. *Percentage of antibiotics bound by 50% serum, determined in two ways*

| Antibiotic | Tube dilution method | Ultrafiltration |
|---|---|---|
| DMCT | 88 | 81 |
| GS | 90 | 78 |
| TC | 58.6 | 58 |
| Ay | 55 | 53 |

*Serum concentrations.* The serum-assay method used for these studies has been described in detail in a previous publication (Roberts et al., 1961); it consists of a serial twofold dilution method using a group A streptococcus as the test organism. Results are expressed both in terms of reciprocals of the serum dilution causing inhibition, and in $\mu$g/ml of each antibiotic in serum. For the latter, representative minimal inhibitory concentrations for the test streptococcus (9282) with the four antibiotics were as follows ($\mu$g/ml): GS, 0.04; DMCT, 0.05; TC, 0.08; and Ay, 0.09. Six volunteers, healthy men and women between the ages of 20 and 30, were used for each of the four studies described below.

1) Single dose study:—Each volunteer, after an overnight fast, took a single dose of a given antibiotic in a four-way crossover study. Breakfast was delayed until 2 hr after ingestion of the antibiotic, and at least 4 days elapsed before any volunteer repeated the study. GS was superior to DMCT, both from the standpoint of absorption and of antistreptococcal activity in the volunteers' serum (Fig. 1 and 2). Ay was quite well absorbed, but even in a dose of 0.6 g it gave the lowest antistreptococcal activity, due to its lower degree of potency. Levels obtained with TC were higher than are usually observed with this antibiotic. Assays of the capsules did not indicate an excessive antibiotic content.

When this study was repeated, with administration of the drug 30 min after breakfast, serum concentrations were considerably lower (Fig. 3). The highest level was under 1 $\mu$g/ml; this was obtained with Ay. TC was influenced more than any of the other antibiotics by food, for reasons that are not readily apparent.

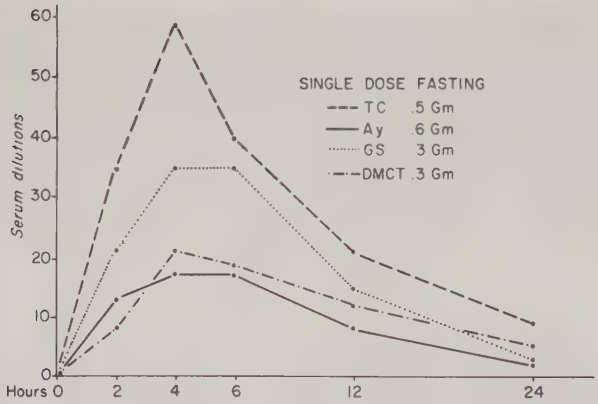


FIG. 1. *Average serum antistreptococcal activity in six volunteers after the ingestion of a single dose of antibiotic in a four-way cross-over study.*

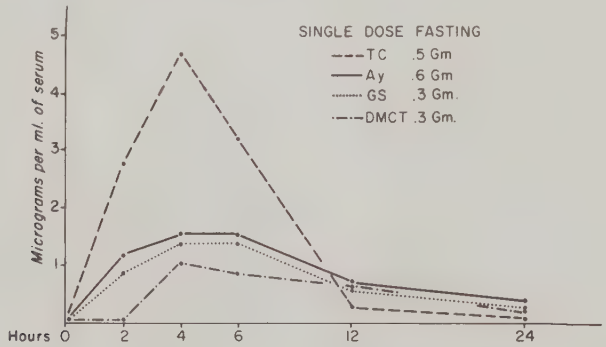


FIG. 2. *Same as Fig. 1, expressed in μg/ml.*

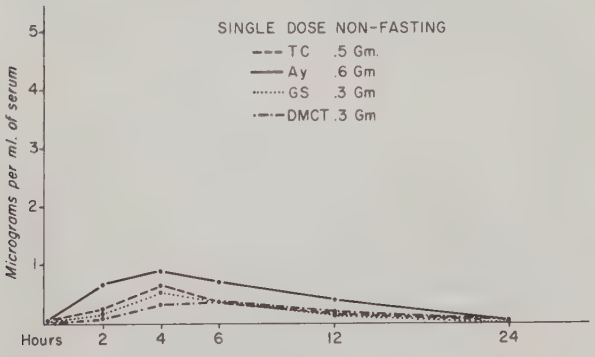


FIG. 3. *Same type of experiment as depicted in Fig. 1 and 2, except that the antibiotics were ingested 30 min after breakfast.*

2) Multiple dose study:—Six volunteers took five doses of the four antibiotics, again in a four-way crossover study, in the amounts listed in Fig. 4 and 5. The doses were spaced 6 hr apart, with the first and fifth being early in the morning with a 9-hr fast. Meals were scheduled so that no capsules were taken within 1 hr of any meal; breakfast was at least 2 hr after the first and fifth doses. Blood was drawn 2, 4, and 6 hr after the first and fifth doses, to see if the relative height of blood levels was altered by multiple doses.

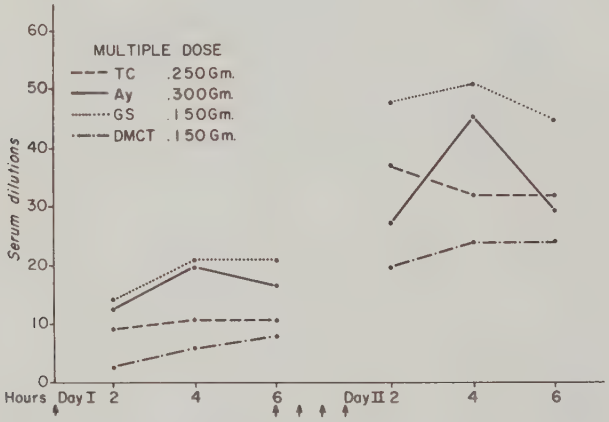


FIG. 4. *Average serum antistreptococcal activity in six volunteers, determined after the first and fifth doses given at 6-hr intervals.*

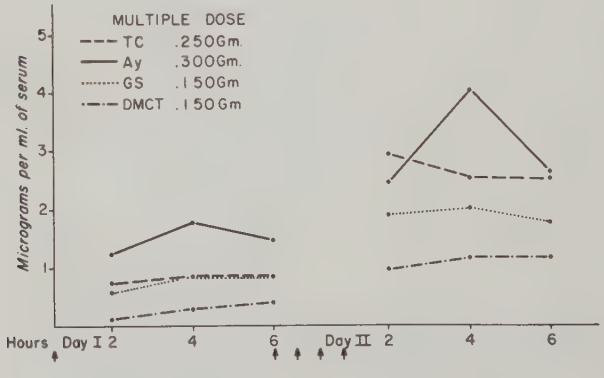


FIG. 5. *Same as Fig. 4, expressed in μg/ml.*

Absorption and serum activity were, as with single doses, better with GS than with DMCT. A significant build-up was noted after the fifth dose with all four drugs, but the magnitude of the increase was no greater with any one drug than with the others. The TC capsules used for this study were not much different in potency than for the single dose study (254 vs. 268 mg), but the blood level values were lower for TC in relation to the other drugs (Fig. 4 and 5). With Ay, both absorption and serum activity were quite good, and compared favorably with the results obtained with TC.

## Discussion

The close agreement in minimal inhibitory concentrations, serum binding, and serum concentrations between GS and DMCT is not surprising in view of their similar chemical structures. The advantages observed in the present study seem to be in favor of GS, particularly in absorption from the intestinal tract. However, because of the small number of volunteers and the variability that can result from differences in potency of the capsules, it is possible that GS is not absorbed to a significantly greater extent. Of interest in this regard are the somewhat divergent results obtained in the single and multiple-dose studies with TC, where capsules from different commercial lots, without greatly different potencies, were employed. The desirability of performing such studies with capsules of uniform potency is apparent, but in the present study commercial supplies of TC and DMTC were deliberately selected as representative of medications being used in clinical practice.

In a previous study (Roberts et al., 1961), the potential usefulness and supposed superiority of DMCT given in smaller doses than TC were questioned on the basis of its toxicity, greater serum binding, and failure to give higher serum activity due to its poorer absorption. These points have all been confirmed in the present study, and are in general also true of GS. The indications that GS may be somewhat superior to DMCT obviously need confirmation on a much larger scale, including evidence concerning toxicity and photosensitivity.

It was of interest that DMCT did not show a greater build-up after the fifth dose than the other tetracyclines, as determined by serum assays over a 6-hr period.

Our data may actually tend to minimize the superiority of GS, since it deteriorates quite rapidly in broth; end points observed after 18 hr incubation may be appreciably less favorable than if the assay method permitted readings at 4 to 6 hr. In clinical practice, where tetracyclines are usually administered every 6 hr, deterioration would presumably not occur to a significant degree.

Although reasonably well absorbed, the lower degree of antibacterial activity of Ay suggests that this antibiotic needs to be given in larger doses to attain clinical efficacy comparable to TC. There is no indication from the present study that this would offer any special advantages. It may well be that this more soluble tetracycline will be useful for intramuscular injections, as proposed by Gradnik, Pedrazzoli, and Ferrero (1960), who are responsible for its widespread use in Italy.

## Literature Cited

Gradnik, B., A. Pedrazzoli, and E. Ferrero. 1960. 4' (Beta hydroxiéthyl) diéthilendiaminométhyl tétracycline: un nouveau dérivé soluble dans le groupe des tétracyclines. Pharm. Acta Helv. 35:529-538.

Roberts, C. E., Jr., D. M. Perry, H. A. Kuharic, and W. M. M. Kirby. 1961. Demethylchlortetracycline and tetracycline: a critical comparison; in vitro activity, serum concentrations and effect of serum binding. A. M. A. Arch. Internal Med. 107:204-211.

# HIGHEST TOLERATED DOSE CONCEPT OF THERAPY WITH ANTIMICROBIAL DRUGS AS EXEMPLIFIED BY THE ADMINISTRATION OF LARGE DOSES OF CHLORAMPHENICOL

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Experience with the highest tolerated doses of penicillin in bacterial endocarditis (Hunter, 1952) and *Proteus* infections (Waisbren, 1953) suggested that intensification of dose might increase the efficacy of other antibiotics. Therefore, 95 patients with severe infections not amenable to usual therapy were given chloramphenicol succinate intravenously in a daily dose that ranged from 6 to 18 g. These cases revealed that this drug, given in the maximal tolerated dose, often gave gratifying results. In addition, they gave data regarding the lack of toxicity of chloramphenicol that should be of aid to clinicians in deciding whether it should be used in high doses in seriously ill patients. The severe infections in which this aggressive approach might be indicated are exemplified by five case reports.

## Materials and Methods

Fifty-eight patients were males and 37 were females. The mean age was 42. Ninety patients were Caucasian, five were Negroes. Seventy-three had no serious previous infection, 22 had a history of one or more serious past infections. Eight had carcinoma and four probably had cirrhosis of the liver. Thirty patients were treated at Milwaukee County General Hospital, and 65 were seen in consultation and treated at one of seven private hospitals in the Milwaukee area. Table 1 shows the types of infection that were treated.

In the majority of patients, the main pathogens were coagulase-positive staphylococci, but severe or protracted gram-negative infections were also included. Forty-two of the patients received gamma-globulin in addition to the chloramphenicol, and 75 received other antibiotics. Previous studies from this (Waisbren, 1957, 1960, 1961) and other laboratories (Knouf, 1958; Stampfli et al., 1959; Schonholtz,

Borgia, and Ritchey, 1959) in regard to the potentiation of antibiotics by gamma-globulin made us feel that this adjunct should be used in the types of patients being treated. Similarly, analysis of our results in the treatment of lung abscesses, and the work of Lepper et al. *(in press)* on staphylococcal bacteremias, showed that severe staphylococcal infections respond better to two antibiotics to which the strain is sensitive, so there was no hesitation in the use of multiple antibiotics in patients who were not doing well (Henschl and Waisbren, 1961). This, of course, often made it impossible to assess the specific role of the high-dose treatment in the clinical result. The rationale for the use of high doses of chloramphenicol in burned patients has been discussed elsewhere (Waisbren, 1961).

TABLE 1. *Infections treated with highest tolerated doses of chloramphenicol*

| Disease | Number of patients | Survivors |
|---|---|---|
| Postoperative wound infection (staphylococcal) | 10 | 9 |
| Severe burns | 16 | 12 |
| Staphylococcal bacteremia | 14 | 10 |
| Staphylococcal osteomyelitis | 12 | 12 |
| Pneumonia (staphylococcal, Friedlanders, undiagnosed) | 7 | 4 |
| Abscesses (abdominal, pelvic, lung) | 15 | 14 |
| Peritonitis | 6 | 6 |
| Meningitis (staphylococcal or *Haemophilus*) | 5 | 5 |
| Endocarditis (staphylococcal or undiagnosed) | 3 | 1 |
| Staphylococcal cellulitis | 5 | 5 |
| Staphylococcal enteritis | 2 | 1 |
| Pyelonephritis | 10 | 9 |
| Salmonellosis | 1 | 1 |
| *Total** | 106 | 89 |

*Total is greater than 95 because some patients fitted into more than one category.

In the majority of instances, the high-dose schedule was started as soon as the patients were seen, since previous studies of chloramphenicol in staphylococcal bacteremias had shown it to be ineffective in usual doses (Waisbren and Abboud, 1960). In many patients, extensive therapy with other antibiotics had been given during previous hospitalizations. A rigid protocol of hematological determinations was not followed, but the mean of each blood determination taken on each day, before, during, and after, was determined. The average dose of chloramphenicol given was 64 g. The highest dose, given to Case 1 of the Case Reports (multiple brain abscesses), was 357 g and the lowest dose was 9 g, given to a 3-year-old girl with staphyloccal bacteremia.

TABLE 2. *Summary of hematologic data on 95 patients who were receiving high doses of chloramphenicol**

| | Before therapy | | | | During therapy | | | | | | | | | | | | | | | | After therapy | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 3rd Day | | 1st Day | | 1st Day | | 3rd Day | | 5th Day | | 7th Day | | 9th Day | | 11th Day | | 13th Day | | 15th Day | | 17th Day | | 19th Day | | 1st Day | | 3rd Day | | 5th Day | | 7th Day | | 9th Day | |
| | N | M | N | M | N | M | N | M | N | M | N | M | N | M | N | M | N | M | N | M | N | M | N | M | N | M | N | M | N | M | N | M | N | M |
| Hemoglobin | 12 | 12.0 | 27 | 12.8 | 53 | 12.9 | 50 | 12.1 | 43 | 12.3 | 34 | 12.0 | 25 | 11.9 | 19 | 11.7 | 15 | 11.7 | 15 | 11.8 | 14 | 12.6 | 13 | 12.1 | 40 | 11.5 | 25 | 11.4 | 20 | 11.9 | 22 | 12.2 | 14 | 11.7 |
| Hematocrit | 9 | 38.8 | 14 | 39.1 | 36 | 40.6 | 43 | 39.9 | 28 | 39.0 | 25 | 38.0 | 21 | 37.5 | 17 | 37.0 | 15 | 38.4 | 11 | 39.5 | 13 | 38.9 | 7 | 35.7 | 25 | 36.8 | 19 | 37.9 | 14 | 38.5 | 15 | 38.9 | 7 | 39.0 |
| W.B.C. in thousands | 8 | 12.1 | 21 | 13.5 | 49 | 13.7 | 45 | 14.0 | 40 | 13.3 | 31 | 12.1 | 25 | 12.7 | 17 | 12.3 | 16 | 15.3 | 11 | 10.5 | 10 | 13.7 | 7 | 11.9 | 36 | 12.5 | 27 | 10.4 | 20 | 11.7 | 19 | 13.0 | 14 | 11.4 |
| Polymorphonuclear leukocytes, % | 10 | 72.0 | 23 | 73.1 | 44 | 76.8 | 49 | 77.9 | 39 | 77.0 | 30 | 76.7 | 25 | 75.8 | 16 | 79.3 | 15 | 77.3 | 16 | 80.8 | 12 | 73.4 | 11 | 73.2 | 37 | 73.4 | 26 | 68.0 | 15 | 75.2 | 20 | 70.0 | 14 | 68.9 |
| Eosinophils, % | 4 | 3.0 | 8 | 2.8 | 14 | 2.8 | 22 | 2.3 | 26 | 2.5 | 19 | 3.3 | 15 | 2.7 | 6 | 1.8 | 11 | 2.8 | 5 | 2.2 | 9 | 3.8 | 8 | 2.6 | 21 | 5.1 | 18 | 3.6 | 9 | 4.6 | 13 | 3.5 | 8 | 4.3 |
| Reticulocytes | | | | | 1 | 5.5 | 3 | .9 | 6 | 1.4 | 4 | .7 | 3 | .5 | 4 | .5 | 3 | .7 | 4 | 1.8 | 1 | .5 | 2 | 1.6 | 4 | 7.5 | 6 | 1.3 | 2 | 1.0 | 4 | 1.1 | 1 | 1.3 |
| Blood iron | | | | | | . | 3 | 147.7 | 1 | 32.0 | 2 | 128.8 | 1 | 187.5 | | | | | | | | | | | 2 | 52.5 | | | | | | | | |

*N=number of determinations; M=mean.

TABLE 3. *Reticulocyte response in a 15-year-old patient who received 114 g* of chloramphenicol*

| Hospital day | 1 | 2 | 3 | 4 | 7 | 10 | 12 | 14† | 18 | 19 | 23 | 24 | 25 | 31 | 37 | 39 | 41 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Dose of chloramphenicol, g | 6 | 6 | 6 | 6 | 6 | 6 | 6 | 6 | 6 | 6 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| Hemoglobin | 14.7 | 17.0 | 17.0 | 12.5 | 17.2 | 17.0 | 16.2 | 17.0 | 8.9 | 10.8 | 12.0 | 13.0 | 14.0 | 13.2 | 15.9 | 14.5 | 13.6 |
| Reticulocyte | – | – | 1.2 | .7 | .4 | .7 | .6 | .7 | .3 | 1.3 | 5.3 | 5.2 | 3.7 | 2.1 | 1.0 | 1.3 | 1.8 |
| Bone marrow aspiration | – | – | – | – | – | – | – | N‡ | – | – | – | – | – | – | – | – | – |

*Six g of chloramphenicol were given from the 1st through the 19th hospital day.

†Two units of whole blood were given on the 14th day.

‡Normal granulopoiesis and erythropoiesis were seen in this specimen.

## Results

*Toleration of high intravenous doses.* The limiting factor in drug toleration was a bitter metallic taste noted when more than 10 g of chloramphenicol was given (dissolved in 500 ml of fluid). When 12 g were given in a single effusion, nausea and vomiting were apt to occur. As much as 18 g were given in a 24-hr period with good toleration, but this was done in two spaced infusions of 9 g each.

No other evidence of drug intolerance was noted, and not a single case of thrombophlebitis occurred from the infusions. There were no skin rashes. The patient in whom the chloramphenicol was injected directly into the cranial cavity showed no toxicity from this mode of administration. No renal or hepatic evidences of drug toxicity were noted.

Throughout, special note was taken of possible toxicity to the hemopoietic system. As many determinations as possible of hemoglobin, reticulocyte counts, blood iron, and white blood counts were obtained. In not a single instance was there evidence of bone-marrow depression, and the pooled data failed to show any depressing effect from the high doses of chloramphenicol used (Table 2). In addition, several cases in whom there was bleeding showed excellent reticulocyte response while patients were receiving 6 to 8 g of chloramphenicol per day (Table 3). Ten bone-marrow specimens aspirated while patients were receiving from 6 to 10 g of chloramphenicol per day revealed no evidence of overt toxicity. The bone-marrow findings reported by Rosenback, Caviles, and Mitus (1960) were not seen in a single instance.

Toleration of gamma-globulin given intravenously (10 ml dissolved in 500 ml of fluid) to 30 of the patients was excellent, in spite of warnings regarding its use in this manner on the package inserts. This is consistent with our experience in the treatment with intravenous gamma-globulin of many other patients.

*Results of therapy.* The specific role of the high doses of chloramphenicol was impossible to evaluate in the cases that received other medications. However, each case was evaluated on the basis of all data available, to assess the value of giving maximal tolerated intensive therapy to patients of this type (Tables 4 and 5); 63 patients showed objective evidence of response to the therapy. There were 21 treatment failures; in 11 instances, the results were indeterminate. In six cases there was definite evidence of effectiveness of high doses of chloramphenicol after lower doses of this drug had failed. Table 5 shows cases in which treatment failure resulted despite the use of chloramphenicol in the high doses. It can be seen that most of these patients received other drugs with no effect.

Bacteriological data were limited, due to distribution of the patients throughout the community; wherever possible, attempts were made to correlate the results of treatment with sensitivity studies.

TABLE 4. *Analysis of results of therapy with highest tolerated dose regimen*

| Therapy | Number of patients | Response | No response | Indeterminate |
|---|---|---|---|---|
| Antibiotics used without gamma-globulin | 27 | 13 | 8 | 6 |
| Antibiotics used with gamma-globulin | 68 | 42 | 12 | 4 |
| High doses of chloramphenicol given after failure with usual doses of chloramphenicol | 8 | 6 | 1 | 1 |
| High doses of chloramphenicol given after failure with usual doses of other antibiotics in same hospital admission | 4 | 4 | 0 | 0 |

The majority of the sensitivity studies that were done were performed at the Milwaukee County General Hospital, where a single tube dilution sensitivity method is used (Abboud and Waisbren, 1959). Correlation between the results of the single tube dilution sensitivity test and the clinical results was possible in 27 patients. Nineteen of 21 patients with sensitive organisms responded; only 3 of 6 with resistant organisms responded. This is a better than usual result with organisms considered resistant in previous studies, and indicates that with high doses reinterpretation of crucial levels of sensitivity may be necessary (Abboud and Waisbren, 1959).

## Exemplary Case Reports

*Case 1 (21-year-old male with multiple brain abscesses). First hospitalization.* Chief complaint: swollen, tender area over frontal sinus. Course: progression of lethargy and fever in spite of daily penicillin (1 million units) and chloramphenicol (1 g); prompt clinical response to daily chloramphenicol, 10 g intravenously (IV) for 10 days, and gamma-globulin (150 ml IV in 10 days). Neurosurgical work-up negative. Discharged from hospital after 3 weeks. *Second hospitalization 6 weeks later.* Chief complaint: headache. Course: localization of lesion in the right parietal region after 10 days observation; drainage of abscess (Joseph Mufson was the intrepid neurosurgeon in this case) and culture of microaerophilic streptococci. Penicillin G (30 million units per day) and erythromycin (2 g per day) given with no clinical response; reoperation with abscess shelled out; no response to high doses of chloramphenicol (10 g per day); third operation with drainage of three more abscesses, with culture yielding microaerophilic streptococci; chloramphenicol continued. Discharged asymptomatic after a total of 232 g of chloramphenicol had been given during a 32-day period. *Third hospitalization 5 days later.* Chief complaint: patient found unconscious, febrile, and with papilledema. Course: two 18-guage needles were inserted in previously made burr holes and catheters threaded into these; 10 ml of a solution containing 50 mg per ml chloramphenicol was injected into these catheters every 4 hr. Chloramphenicol (8 g) and gamma-globulin (20 ml) were given IV daily. There was a prompt clinical response. Parenteral therapy and injections into the intracerebral catheters were continued for 1 month, when the antibiotic was changed to erythromycin (0.5 g per os 4 times a day), which was taken for the past 18 months with no recurrence of difficulty. At no time during the three hospitalizations was evidence of drug toxicity found. In all, 357 g of chloramphenicol, 400 million units of penicillin G, and 400 ml of gamma-globulin were given intravenously, and 20 g of chloramphenicol were injected directly into the brain without evidence of toxicity.

TABLE 5. *Analysis of cases in which high doses of chloramphenicol failed*

| Diagnosis | Total CMC* used, g | Other therapy unsuccessful | Other therapy successful | Outcome |
|---|---|---|---|---|
| Bacterial endocarditis | 68 | Terramycin<br>Streptomycin<br>Penicillin | None | Death |
| Aspiration pneumonia (*Pseudomonas*) | 86 | Polymyxin B<br>Neomycin<br>Penicillin<br>Oxytetracycline | None | Death |
| *E. coli* septicemia | 26 | Penicillin | None | Death |
| Bronchopneumonia (acute) | 24 | Penicillin<br>Neomycin<br>Erythromycin | None | Death |
| Wegener's granulomatosis | 36 | Novobiocin<br>Kanamycin<br>Vancomycin<br>Isoniazid<br>Streptomycin<br>Hydrocortisone | None | Death |
| Bacterial endocarditis | 18 | Streptomycin<br>Penicillin<br>Ristocetin | None | Death |
| *Klebsiella* pneumonia | 8 | Streptomycin | None | Death |
| Wegener's granulomatosis | 30 | Cytoxan<br>Penicillin | None | Death |
| *Pseudomonas* infection after thoracotomy | 90 | None | Polymyxin B | Cure |
| Lupus erythematosus | 20 | Ilosone | Cortisone | Some response to steroids, but still febrile |
| Probable collagen disease | 44 | Tetracycline<br>Isoniazid | Hydrocortisone | Some response. Diagnosis still questionable |
| Squamous cell carcinoma with secondary infection | 38 | Methicillin<br>Penicillin<br>Erythromycin | Surgery | Patient became afebrile |
| Probable collagen disease | 60 | Penicillin<br>Methicillin<br>Gamma-globulin | Steroids | Some response |
| Ascending cholangitis | 54 | Erythromycin<br>Nitrofurantoin | None | Death |
| Carcinoma of esophagus with mediastinitis | 72 | Dehydro-streptomycin | None | Death |
| Viral encephalitis | 124 | Penicillin<br>Streptomycin<br>Erythromycin | Steroids | Some response to steroids |
| Post-traumatic septic arthritis | 34 | None | Novobiocin<br>Vancomycin | Cure |
| Esophageal ulcer with hemorrhage | 28 | Penicillin<br>Dehydrostreptomycin | None | Death |
| Lupus erythematosus | 60 | None | Steroids | Response |
| Burns, 3rd degree | 96 | Methicillin<br>Penicillin | None | Death—not due to infection |
| Burns, 3rd degree | 102 | Methicillin<br>Penicillin | None | Death—not due to infection |

*CMC=chloramphenicol.

*Case 2* The patient was a 37-year-old Caucasian woman with generalized staphylococcal pneumonia and septicemia. She responded to intensive therapy with chloramphenicol after failure of vigorous treatment with methicillin, chlortetracycline, kanamycin, and moderate doses of chloramphenicol.

Onset of illness was an otitis media, from which was cultured a coagulase-positive staphylococcus. Progression occurred during a 12-day period in which both erythromycin and novobiocin were being given by mouth. The patient was hospitalized when a palsy of the seventh nerve developed on the right. Admission X rays showed a patchy infiltration of the left lung field. During the next 18 days there was spread of the lung lesion and the patient became increasingly septic, in spite of therapy with penicillin G (78.4 million units), methicillin (67 g), streptomycin (5 g), chlortetracycline (4 g), gamma-globulin (20 ml), and cortisone (200 mg) (Fig. 1). When seen on the 19th hospital day the patient was cyanotic, had grunting respirations, and appeared mortally ill. Temperature was 104 F, pulse 140, hemoglobin 10.2, white count 28,000 with 96% polymorphonuclear leukocytes.

The following medications were given immediately: chloramphenicol, 8 g dissolved in 500 ml of 5% dextrose in water; hydrocortisone, 500 mg in 1 liter of 5% dextrose in water (cortisone had been started the day previously, so it was felt necessary to continue it); gamma-globulin, 20 ml dissolved in 500 ml of 5% dextrose in water. There was a clinical response to this program and the patient was afebrile within 24 hr. The cortisone was tapered off and discontinued in 8 days. A total of 121 g of chloramphenicol and 175 ml of gamma-globulin was administered during the next 16 days. Anemia was present during the entire hospital stay, but the reticulocyte count remained at2.7% and two bone-marrow examinations were interpreted as normal. At the time of discharge, the lungs were clear, the nerve palsy had remitted, and the patient has remained asymptomatic (Fig. 2).

*Case 3.* This case represents a probable staphylococcal endocarditis that responded to the addition of high doses of chloramphenicol to the therapeutic regimen. The patient was a 59-year-old Negro woman who, when first seen, had been in the hospital for 16 days with fever and a grade III systolic mitral heart murmur. She was anemic, hypotensive, had hematuria, and was irrational in spite of prior therapy with 19.2 million units of penicillin G and 12 g of streptomycin. Blood urea nitrogen was 65 mg %. During the next 48 hr she received 1 g of vancomycin intravenously, 10 g of methicillin intravenously, 8 g of methicillin intramuscularly, 20 million units of penicillin G, and 20 ml of gamma-globulin intravenously. In spite of this her temperature rose and remained at 103 F, her pulse was 140 beats per min, blood pressure remained at 80/40 with Levophed, she was comatose, and her white blood count was 15,900 with 91% polymorphonuclear leukocytes.

Due to the apparent failure of the antibiotics being used, 8 g of chloramphenicol, given intravenously, were added to the therapeutic program. The patient was afebrile and alert the next day and her blood pressure stabilized at 90/70 without Levophed. Thereafter the following intravenous medications were given for 14 days for a presumptive diagnosis of staphylococcal endocarditis; chloramphenicol, 8 g; gamma-globulin, 10 ml; penicillin G, 6 g; methicillin, 10 g; and vancomycin, 0.5 g. At the end of 14 days, upper extremity cut-down sites no longer were available, and it was decided to stop all therapy for a period of observation. During the next 6 months the patient remained afebrile and gradually improved although at the time of this writing she continues to have intermittent heart failure. Which of the drugs used is to be given credit for the ultimate cure of this case remains doubtful, but it is of interest that clinical control of infection did not occur until the high doses of chloramphenicol were added to the therapeutic program.

*Case 4.* In this patient subacute pyelonephritis was apparently cured. The patient was a 37-year-old Caucasian male who, during a 4-month period, had three attacks of acute pyelonephritis due to *Escherichia coli* in spite of two 10-day courses of chloramphenicol (1 g per day) and one 10-day course of nitrofurantoin (200 mg per day). At the onset of the fourth attack he was hospitalized. Intravenous pyelograms were normal and culture of his urine showed one million *E. coli* in each specimen of a 2-glass sample. These bacteria were sensitive to a concentration of 1.5 μg/ml of chloramphenicol. A 1-week course of chloramphenicol, 8 g intravenously in 1.5 liters of 5% dextrose in water, and gamma-globulin, 10 ml in 500 ml of saline, was given without incident. There was an immediate clinical response, and urine cultures became sterile and have so remained for the 18 months he has been followed since.

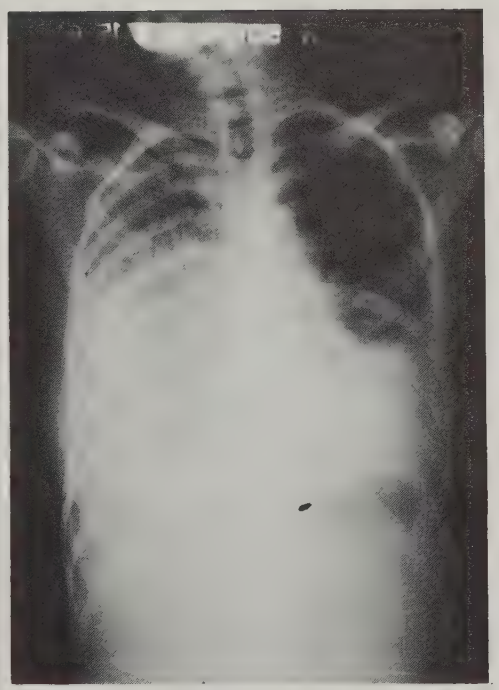

FIG. 1. *Chest roentgenogram of a 37-year-old woman after 10 days of treatment with methicillin, chlortetracycline, and kanamycin.*

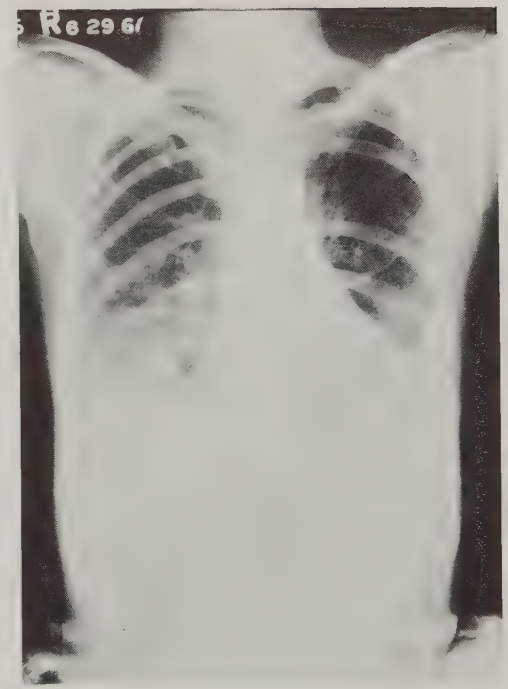


FIG. 2. *Chest roentgenogram of patient in Fig. 1, 4 weeks later, after response to therapy with high doses of chloramphenicol.*

*Case 5.* This patient had ulcerative colitis with overwhelming sepsis. Massive doses controlled infection and allowed definitive surgery. The patient was a 19-year-old Caucasian girl; ulcerative colitis had progressed in spite of a partial colectomy, diet, cortisone, and nonabsorbable sulfa drugs. She was admitted to the hospital for total colectomy, but became febrile shortly after admission. She became progressively more toxic in spite of therapy with penicillin G, 10 million units, chloramphenicol, 2 g, and novobiocin, 7 g. When seen on the tenth hospital day, she was unresponsive, her temperature was 104 F, her hemoglobin was 8 g, her white blood count was 13,000 with 98% polymorphonuclear leukocytes, and cultures of her blood, urine, and stool showed coagulase-positive *Staphylococcus aureus*. Therapy was instituted as follows: chloramphenicol, 8 g; methicillin, 4 g; gamma-globulin, 20 ml; all were given intravenously daily. In addition, she was given 14 units of plasma and 4 units of blood during the next week. The patient became afebrile and responsive in the next 24 hr, but developed jaundice that was interpreted to be due to the hemolytic staphylococcus. The choramphenicol, methicillin, and gamma-globulin were continued in the same dosage for 7 days, during which steady improvement occurred. Chloramphenicol was then reduced to 6 g per day and the three drugs were continued for 22 days; then her condition was deemed good enough for surgery and a Miles resection and iliostomy were performed. During this procedure, several intra-abdominal sterile abscesses were drained. Postoperative course was uneventful. The patient left the hospital weighing 64 pounds and 1 year later was seen in the clinic with a weight of 84 pounds. In all, during 8 weeks of hospitalization, she received 141.5 g of chloramphenicol, 80 g of methicillin, 250 ml of gamma-globulin, 32 units of plasma, and 16 units of whole blood without evidence of toxicity.

These cases do not illustrate the entire spectrum of situations in which maximal tolerated therapy has been found useful, but should give some idea of the type of infection in which this aggressive approach may be indicated.

## Discussion

In view of recent editorial comment (Damashek, 1960), the choice of chloramphenicol for the testing of the highest tolerated dose concept may seem strange. However, a consideration of the toxicity of other agents that might have been tried reveals that, in fact, chloramphenicol is the logical choice. Renal toxicity at the usual dose precludes neomycin (Waisbren and Spink, 1950), streptomycin, polymyxin B (Hopper, Jawetz, and Hinman, 1953), bacitracin, and vancomycin (Waisbren, 1958; Waisbren et al., 1960). Hematological difficulties that have been correlated with high doses make ristocetin an unlikely choice (Gangarosa, Johnson, and Ramos, 1960). The tetracycline group of antibiotics cannot be tolerated in large doses intravenously because of the development of an almost invariable thrombophlebitis as well as nausea and vomiting. This leaves novobiocin, erythromycin, and chloramphenicol as likely choices. Frequent development of skin rashes with novobiocin would make this drug difficult to use in many cases (Finland and Nichols, 1957). Erythromycin would seem a justifiable drug to try in certain instances, but it is not always tolerated well in high intravenous doses, and the development of resistance during therapy occurs frequently (Waisbren and Strelitzer, 1959).

The fact that there was no significant toxicity in this group of 95 patients given high doses of chloramphenicol might have been

expected, when one considers that most reports of hematological toxicity of chloramphenicol are isolated instances culled from millions of patients who have received this drug (Damashek, 1960; Lewis et al., 1952).

The study of Krakoff, Karnofsky, and Burchenal (1955) of four patients given high doses of chloramphenical showed three to develop severe glossitis and one bone-marrow depression. All of our patients were given 1000 $\mu$g of vitamin $B_{12}$ and 5 mg of folic acid daily, because of our previous experience that this prevented "antibiotic glossitis." The possibility must be mentioned that these vitamins also might have prevented toxicity to the bone marrow. Rosenback et al. (1960) indicated that chloramphenicol not infrequently has an effect on the bone marrow; our data show that this effect, if it occurs as often as might be inferred from their article, is not harmful to the patients.

The use of gamma-globulin in many of the patients is consistent with the thesis of maximal tolerated therapy, but no attempt was made to assess its particular value in this group, in view of other confirmatory studies (Stampfli et al., 1959; Bringhurst and Marcus, 1961). However, the excellent toleration of gamma-globulin given by the intravenous route (dissolved in 500 ml of fluid) in 30 of the patients is worthy of comment, due to the fact that its labeling interdicts usage by this route. Reason for this is not clear, but it may have been based on the opinion of Gross, Gitlin, and Janeway (1959) for which we can find no confirmatory data. In the administration of gamma-globulin to hundreds of patients we have seen only one generalized reaction and that was after an intramuscular injection in a patient who previously had a slight reaction to an intramuscular injection.

In a sense it is unfortunate that it was necessary to select a controversial drug such as chloramphenicol to test the thesis of highest tolerated dose, because this might tend to obscure the main point that, in therapy of infectious diseases, drugs may have to be given to the point of toxicity before a clinical response is obtained. In other situations, such as the chemotherapy of cancer and the use of corticoids in lupus nephritis, this philosophy seems to have been more generally accepted (Schell and Hall, 1958; Pollak, 1960). In the last analysis, the individual clinician will have to continue to decide on drug dosage on the basis of the natural history of the disease, its prognosis, the condition of the patient, and the efficacy and toxicity of the drug or drugs being used. This study, which offers data on the lack of toxicity of large doses of chloramphenicol, should be helpful in rationalizing therapy for the patients who might be aided by large doses of this antibiotic. However, if the report but causes a reconsideration of the rigid dose schedules often used in the treatment of infections, it will have served its main purpose.

## Literature Cited


Abboud, F., and B. A. Waisbren. 1959. Correlation between *in vitro* studies and response to antibiotic therapy in staphylococcic bacteremia. A. M. A. Arch. Internal Med. **104**:226-233.

Bringhurst, M., and S. Marcus. 1961. Antibacterial action of penicillin in combination with serum globulins. J. Lab. Clin. Med. **57**:874-882.

Damashek, W. 1960. Chloramphenicol--A new warning. J. Am. Med. Assoc. **174**:1853-1854.

Finland, M., and R. L. Nichols. 1957. Novobiocin. Antibiotica et Chemoterapia **4**:209-392.

Gangarosa, E. J., T. R. Johnson, and H. S. Ramos. 1960. Ristocetin induced thrombocytopenia: site and mechanism of action. A. M. A. Arch. Internal Med. **105**:89-99.

Gross, P. A. M., P. Gitlin, and C. A. Janeway. 1959. The gamma globulins and their clinical significance. IV. Therapeutic use of gamma globulins. New Engl. J. Med. **260**:170-177.

Henschl, E., and B. A. Waisbren. 1961. Lung abscess in a general hospital. Diseases of the Chest **40**:625-630.

Hopper, J., E. Jawetz, and F. Hinman. 1953. Polymyxin B in chronic pyelonephritis: observations on the safety of the drug and its influence on the renal infection. Am. J. Med. Sci. **225**: 402-409.

Hunter, T. H. 1952. Treatment of some bacterial infections of the heart and pericardium. Bull. N. Y. Acad. Med. **28**:213-228.

Knouf, E. G. 1958. The combined use of gamma globulin and chloramphenicol in the treatment of refractory infections. Antibiotics Ann. 1957-58, p. 585-597.

Krakoff, I. H., D. A. Karnofsky, and J. H. Burchenal. 1955. Effects of large doses of chloramphenicol on human subjects. New Engl. J. Med. **253**:7-10.

Lewis, C. N., L. E. Putnam, F. D. Hendricks, I. Kerlan, and H. Welch. 1952. Chloramphenicol (Chloromycetin) in relationship to blood dyscrasias with observations on other drugs. Antibiotics & Chemotherapy **2**:601-609.

Pollak, V. E. 1960. Lupus nephritis: the effect of large doses of prednisone on the progression of the disease. Proc. 11th Ann. Conf. Nephrotic Syndrome.

Rosenback, L., H. P. Caviles, and W. J. Mitus. 1960. Chloramphenicol toxicity: Reversible vacuolization of erythroid cells. New Engl. J. Med. **263**:724-728.

Schell, R. F., and B. E. Hall. 1958. Experience with triethylene melamine (TEM) and triethylene thiophosphoramide (Thio TEPA) in recurrent or metastatic solid tumor. Surg. Gynecol. Obstet. **106**:459-465.

Schonholtz, F. J., C. A. Borgia, and S. J. Ritchey. 1959. The combined use of gamma globulin and broad-spectrum antibiotics in the treatment of osteomyelitis. Antibiotics Ann. 1958-59, p. 635-642.

Stampfli, V. K., G. A. Spengler, S. Barandun, and G. Riva. 1959. Die Therapie bacterieller Infektionen mit γ-globulin Praparaten. Helv. Med. Acta **26**:424-460.

Waisbren, B. A. 1953. Treatment with large doses of penicillin in case of severe bacteremia due to Proteus. A. M. A. Arch. Internal Med. **91**:138-141.

Waisbren, B. A. 1957. The treatment of bacterial infections with the combination of antibiotics and gamma globulin. Antibiotics & Chemotherapy **7**:322-333.

Waisbren, B. A. 1958. Current concepts in therapy. New Engl. J. of Med. **258**:1213-1215.

Waisbren, B. A. 1960. Pyogenic osteomyelitis and arthritis of the spine treated with combinations of antibiotics and gamma globulin. J. Bone and Joint Surg. **42**:414-429.

Waisbren, B. A. 1961. The medical approach to patients with severe burns. Wis. Med. J. **60**:475-480.

Waisbren, B. A., and F. Abboud. 1960. Bacteremia due to coagulase-positive *Staphylococcus aureus*. Ann. Internal Med. **52**:643-676.

Waisbren, B. A., L. Kleinerman, J. Skemp, and G. Bratcher. 1960. Comparative clinical effectiveness and toxicity of vancomycin, ristocetin, and kanamycin. A. M. A. Arch. Internal Med. **106**:179-193.

Waisbren, B. A., and W. W. Spink. 1950. A clinical appraisal of neomycin. Ann. Internal Med. **33**:1099-1119.

Waisbren, B. A., and C. L. Strelitzer. 1959. Antibiotic sensitivities of staphylococci isolated before and after patients were given antibiotics. Am. J. Med. Sci. **238**:202-210.

# PREVENTION OF RECURRENT RHEUMATIC FEVER IN CHILDREN AND ADULTS

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The effectiveness of various prophylactic agents in preventing recurrences of acute rheumatic fever by diminishing the risk of acquiring group A streptococcal infections has been demonstrated in a number of studies (Mou, Feldman, and Hartenstein, 1960). There are, however, some differences of opinion regarding the optimal duration and type of prophylaxis. More importantly, we are lacking information on the course of rheumatic heart disease under continuous prophylaxis. The present report is part of a long term study which is designed to answer some of these questions.

It is evident from other studies that oral and repository (benzathine) penicillin, and sulfonamides are highly satisfactory agents for the prevention of group A streptococcal infections. Because some patients are allergic to these drugs, we have sought to evaluate other antibiotics that might be used as substitutes for them. In this report the effectiveness, acceptability, and toxicity of oral and repository penicillin will be compared with triacetyloleandomycin and two forms of erythromycin, the erythromycin propionate ester and the erythromycin propionate lauryl sulfate ester. Additional data on the life history of some rheumatics, after 6 years of prophylaxis, are included.

## Materials and Methods

Patients are admitted to the Prophylaxis Clinic if they have rheumatic heart disease or a definite history of an episode of rheumatic fever. Clinic sessions are held weekly, but each patient attends once a month on the same relative day. Throat and nose cultures and blood specimens are obtained at each monthly visit. Throat swabs are plated directly onto sheep's blood agar and antistreptolysin-O (AS-O) is measured by a spectrophotometric method devised in this laboratory. The benzathine penicillin G (both oral and intramuscular) and the triacetyloleandomycin were supplied by Edward F. Roberts, Wyeth Laboratories. The erythromycin salts were provided by R. S. Griffith, Eli Lilly and Company.

Alternate patients are placed in the intramuscular group and receive 1,200,000 units of benzathine penicillin G at each monthly

visit. The second group was provided with a single 200,000 unit tablet of benzathine penicillin G each morning during the initial 41 months of the clinic. In the fall and winter of the fourth year, patients on oral medication received either the benzathine penicillin G or triacetyloleandomycin (250 mg each day) in a double blind comparison of the two agents. During the past 23 months, the oral group has received erythromycin propionate ester (250 mg daily) for a 9-month period, and erythromycin propionate ester lauryl sulfate for the remaining 14 months.

Altogether, 188 persons have been observed for 3 to 72 months, or a total of 4,893 patient months. Of these, 60% are females; the age range is from 4 to 74 years, with 35% less than 20 years old. The racial composition of the clinic is: Caucasian, 157 (84%); Negro, 27 (14%); and Indian, 4 (2%). Six patients have recovered from an episode of subacute bacterial endocarditis, and 16 have had cardiac surgery. These 22 patients represent 12% of the clinic population.

Of the 188 patients, 54 (29%) have had more than one episode of acute rheumatic fever prior to entering the clinic. A single attack occurred in 134 (71%) which includes almost all of the younger patients who have been referred immediately following their initial episode. Those with two (17%) or three, attacks (7%) represent almost a quarter of the total clinic population.

## Results and Discussion

There has been no instance of recurrent acute rheumatic fever detected in any patient who has remained in the clinic. During the 6 years of this report period, group A streptococci were isolated only twice from 4,893 cultures. One patient, a 26-year-old woman, was receiving oral benzathine penicillin G, but the positive culture occurred after the patient had missed a clinic and had been without medication for a month. The other, a 13-year-old girl, had a positive throat culture during the period she had been taking erythromycin lauryl sulfate ester. AS-O titers showed significant increases in both patients in the month after the positive streptococcal culture.

The AS-O data from the six treatment groups have been combined, since no major quantitative differences have been noted in relation to age, sex, or drug. There has been a progressive fall in the median AS-O value for the entire clinic population from a high of slightly more than 2,000 to slightly less than 500 at the end of the 6 years. (These values are about five times higher than Todd units.)

The median AS-O values, of a group of 42 patients who were admitted to the clinic during its first 3 years and who had an initial median AS-O titer of 3,200, are of special interest. This decreased to less than 1,000 units after 36 months of prophylaxis and to 500

units for those patients who have continued to attend the clinic for 60 months or more.

There were 12 possible drug reactions during the 6-year observation period. Transient hives were reported by four patients, three while on repository penicillin (among 2,232 injections) and one, while receiving sulfamethoxypyridazine (69 patient months). Minor skin rashes were reported by one of the "intramuscular" subjects, two of the oral penicillin subjects (1,180 patient months), and one of the triacetyloleandomycin (181 patient months) subjects. Other reactions to triacetyloleandomycin included gastro-intestinal distress in three patients and severe headaches on two separate occasions in another patient. Both erythromycin preparations have been noteworthy with regard to the apparent complete absence of toxic manifestations or reported side effects (1,231 patient months).

The concept of preventing recurrent acute rheumatic fever with continuous antibacterial therapy to prevent new group A streptococcal infections is well established. There are, however, differing opinions regarding the duration of such continuous prophylaxis. It has been suggested, in fact, that early antibiotic therapy of every sore throat is sufficient to prevent recurrent rheumatic fever. Unfortunately, such infections are often not clinically apparent, making this a hazardous control method.

Data collected in our laboratory, in a continuing study of the prevalence of group A streptococcal types in Syracuse, New York, indicates that between 1950 and 1960, 26 group A streptococcal types were identified, but more than 90% of the isolations could be accounted for by six types. This suggests that the turnover of streptococcal types is relatively slow in this community (probably in others, as well) and that the rheumatic has a limited opportunity to acquire natural immunity to many types or to have many recurrences. Available types may be "used up" within a few years. The relative ease, safety, and inexpensiveness of prophylaxis lead us to maintain rheumatics on permanent prophylaxis.

The continuing decline of the median AS-O values for the entire clinic population, despite the introduction of new patients throughout the 6-year period, suggests that prophylaxis not only prevents clinical disease but reduces the number of subclinical infections, as well. This is supported by the experiences of three families who are participating in a family respiratory disease study, being conducted by this department. The three mothers (ages 25, 33, and 37 years, respectively) are also patients in the Rheumatic Fever Prophylaxis Clinic. Even though there have been repeated instances of group A streptococcal isolations from these families during the past three years, the mothers have never had a positive culture for group A streptococci or increases in their AS-O titers.

The continuous decline in the median AS-O titers of the 42 patients who had markedly elevated titers on admission is of marked

significance and suggests that it is a reliable indicator of the efficacy of the prophylaxis regimens. Continuous prophylaxis certainly appears to be preventing almost all (or all) subclinical and clinical streptococcal infections, thereby, inhibiting the immunological response that often accompanies the rheumatic fever recurrence. Whether it is better for the patient to be converted to an immunological "infant" by continuous prophylaxis, unfortunately, cannot be answered from this data.

All five treatment regimens appeared to be effective in preventing rheumatic fever, since there were no recurrences. The data of others, such as Feinstein, Wood, and Epstein, (1959) and Wright et al. (1959) suggest that repository penicillin is the superior streptococcal prophylactic agent. Oral penicillin and triacetyloleandomycin both appeared to be effective in our clinic, but the occurrence of several mild toxic reactions with the latter drug might limit its usefulness somewhat. The erythromycin propionate esters were completely free of undesirable side effects, but there was one isolation of group A streptococci from a patient that we are certain was taking the medication. These, however, would appear to be useful drugs for the penicillin sensitive patient.

In summary, 188 post-rheumatic fever patients have been followed for a 6-year period on six different streptococcal prophylaxis regimens. There have been no recurrences of rheumatic fever, an extremely rare positive culture for group A streptococci, and a persistent decrease in the median AS-O level of the entire clinic population.

## Acknowledgments

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## Literature Cited

Feinstein, A. R., H. F. Wood, and J. A. Epstein. 1959. A controlled study of three methods of prophylaxis against streptococcal infection in a population of rheumatic children. II. Results of the first three years of the study including methods for evaluating the maintenance of oral prophylaxis. New Engl. J. Med. **260**:697-702.

Mou, T. W., H. A. Feldman, and H. Hartenstein. 1960. Prevention of recurrent rheumatic fever in children and adults with oral and repository penicillin. Am. J. Med. Sci. **239**:403-416.

Wright, W. W., H. Welch, J. Wilner, and E. Roberts. 1959. Body fluid concentrations of penicillin following intramuscular injection of single doses of benzathine penicillin G and/or procaine penicillin G. Antibiotic Med. & Clin. Therapy **6**:232-241.

# PROLONGED ADMINISTRATION OF ANTIBIOTICS IN PATIENTS WITH CHRONIC BRONCHIAL DISEASE

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The use of antibiotics has appeared to be beneficial in the acute bacterial infections of the lower respiratory tract of patients with chronic bronchial disease. Several early studies suggested that prolonged treatment with various drugs in varying doses would ameliorate the symptoms of the disease. It was not clear whether this effect was solely the prevention of the acute complicating infections or whether there was a desirable effect on the course of the underlying disease. In terms of changes in microbial flora of the sputum most closely related to a favorable course, the mechanism by which the drugs act was unsettled.

The present study was begun in a large group of patients to evaluate the effect, over long time periods, of orally administered antibiotics (tetracycline, penicillin, and an oleandomycin-penicillin combination) in controlling the pulmonary infections that may occur in these diseases. The results were evaluated not only by clinical criteria but also by frequent bacteriological examinations of the sputum, tests of pulmonary function, and serum proteins. The present report covers the first 3 years of the study, during which 98 patients were divided in a double-blind manner into one of four therapeutic groups. Data for the first half of this study have been reported (Cherniack et al., 1959).

## Materials and Methods

Patients were included in this study if bronchography revealed one or more areas of bronchiectasis or if they had had a history of chronic productive cough for one or more years and repeated lower respiratory tract infections, without another reason for the cough and sputum.

As patients entered the study they were assigned in rotation to one of our treatment groups. Occasionally a patient was placed out

of the strict order of rotation in order to make the groups more nearly uniform with respect to sex, age, and complications such as hypogammaglobulinemia. All placements were made by one of us who had not examined the patients previously and who did not make bacteriological examinations of the sputum or tests of pulmonary function, and did not participate in the evaluation of the clinical status of patients when they returned to the clinic.

All of the other participants in the investigation and all of the patients were unaware of what drug the patient was receiving.

One group of patients received 2 g of tetracycline daily in four divided doses 30 min before each meal and at bedtime. During the first year, tetracycline hydrochloride with citrate buffer was substituted. A second group received 1 g of potassium penicillin G a day (1,600,000 units) in four divided doses; the third group received 2 g of a combination of oleandomycin and penicillin (1.3 g of oleandomycin and 0.7 of penicillin) a day in four divided doses. The fourth group received a sucrose placebo four times a day. All medications were dispensed in capsules of identical shape, size, and color. The Chas. Pfizer and Co., Inc., Brooklyn, N.Y., and the Lederly Laboratories Division, American Cyanamid Company, Pearl River, N.Y., provided the antibiotics and placebos used in this study. Every patient was started on eight of these capsules each day. If an acute respiratory illness required antibiotic therapy, the usual therapy was discontinued and the patient was given the therapeutic regimen that appeared to be indicated, either as an out-patient or after hospitalization. The physician in charge of the patient during this illness was not informed as to which antibiotic the patient had been receiving. At the termination of the episode the patient was returned to his original treatment group.

One or more sputum samples were collected and treated in the following manner. Sputum was homogenized in sterile isotonic sodium chloride solution and cultures made. A fleck from the homogenized suspension of sputum was washed three times in isotonic saline, and cultures were also made from this. Both samples were cultured on the following media: human blood agar, penicillin blood agar (1 unit of penicillin per milliliter), eosin-methylene-blue agar, and Sabouraud's agar. All blood media were inoculated with a streak of *Staphylococcus* to aid in the isolation of *Haemophilus influenzae*. Duplicate cultures were made on all media except Sabouraud's; one culture was incubated aerobically. Organisms were identified by standard bacteriological methods, and the relative numbers of each organism isolated were recorded. When possible, *Diplococcus pneumonia* (pneumococci) and $\beta$-hemolytic streptococci were grouped, with the sera available. Coagulase tests were done on staphylococci, and coagulase-positive staphylococci were bacteriophage-typed, tested for the production of $\alpha$-hemolysin, and tested for sensitivity to penicillin, erythromycin, tetracycline, and oleandomycin. *H. influenzae*

organisms were also tested for sensitivity to the above four antibiotics. The cultures were saved by weekly transfers in Levinthal medium.

After therapy was begun, the patients were seen at monthly intervals. At each visit a blood sample was taken and sputum collected. A history was obtained with particular attention to respiratory illnesses and possible side effects of the drug used, and the patient was weighed and examined. Estimates by patients of their daily sputum volume, given at each visit, were checked against 24-hr collections for several months during the course of this study.

A patient was considered to have had a respiratory tract illness when he gave a history of an illness with symptoms predominantly involving the respiratory tract, either an acute exacerbation of previous symptoms or the appearance of new symptoms. When a patient complained of the acute appearance of dyspnea, without other respiratory symptoms, this was not considered to be a respiratory tract illness. Respiratory illnesses were classified as affecting the lower respiratory tract if the illness was associated with an increase in cough and sputum.

Thermometers were distributed to the patients and they were instructed in their use. A patient was considered to have fever if his oral temperature exceeded 100 F one or more times a day.

## Results

The 98 patients were treated for six or more months. A total of 23 were in the placebo group for an aggregate of 465 patient-months. The figures for penicillin were 26 patients and 420 months; for oleandomycin-penicillin, 24 patients and 450 months; and for tetracycline, 25 patients and 474 months. Some patients dropped out of the study because of social changes such as retirement or a move from city, but only two were lost because of drug intolerance, one in the placebo group and one in the oleandomycin-penicillin group. The details of the adverse effects are reported elsewhere (Sobel et al., *to be published).*

Analysis of the status of the patients, prior to entering into the study, revealed that the groups were similar in age, distribution, degree of pulmonary functional disability, and duration and type of illness. Most of the patients had X-ray evidence of bronchiectasis. In Table 1 the groups are compared in terms of the variables used in this study. It can be seen there were only minor variations in amount and nature of sputum, prior history of morbidity, and the nature of the microbial flora of the sputum. None of these differences was significant.

TABLE 1. *Status of patients prior to entry to protocol*

| Characteristics before treatment | Drug group | | | |
|---|---|---|---|---|
| | Placebo | Penicillin | Oleandomycin-penicillin | Tetracycline |
| Average sputum volume (ml/24 hr) | 84 | 83 | 70 | 53 |
| Sputum color | | | | |
| White | 9 | 7 | 10 | 7 |
| Yellow | 9 | 11 | 8 | 12 |
| Green | 4 | 4 | 5 | 3 |
| Bloody | 0 | 1 | 0 | 1 |
| Episodes lower respiratory disease per patient per year | 3.4 | 3.4 | 2.5 | 3.7 |
| Microbial flora in sputum | | | | |
| *Haemophilus influenzae* | | | | |
| Negative | 12 | 10 | 12 | 12 |
| Positive 50% | 2 | 2 | 0 | 2 |
| Positive 50% | 5 | 9 | 6 | 6 |
| Coagulase-positive staphylococci | | | | |
| Negative | 12 | 16 | 13 | 15 |
| Positive 50% | 2 | 3 | 3 | 3 |
| Positive 50% | 5 | 2 | 2 | 2 |
| Pneumococcus | | | | |
| Negative | 18 | 18 | 13 | 14 |
| Positive 50% | 0 | 1 | 0 | 1 |
| Positive 50% | 1 | 2 | 5 | 5 |
| *Klebsiella* | | | | |
| Negative | 15 | 18 | 18 | 17 |
| Positive 50% | 0 | 1 | 0 | 2 |
| Positive 50% | 4 | 2 | 0 | 1 |
| *Pseudomonas* | | | | |
| Negative | 15 | 14 | 12 | 15 |
| Positive 50% | 2 | 2 | 5 | 3 |
| Positive 50% | 2 | 5 | 1 | 2 |
| *Proteus* | | | | |
| Negative | 16 | 19 | 16 | 18 |
| Positive 50% | 0 | 1 | 1 | 0 |
| Positive 50% | 3 | 1 | 1 | 2 |

Several of the clinical manifestations which were evaluated have been listed in Table 2. It can be seen that there was some reduction in sputum volume in all three of the drug-treated groups, compared to the placebo-treated patients, but only with tetracycline was the difference of significant magnitude. The tetracycline patients also started with a somewhat lower volume. It is noted that the average sputum volume increased somewhat in all groups. This probably reflects the fact that most patients entered the study immediately after hospitalization and vigorous therapy. More sputum samples were colorless or white among the oleandomycin-penicillin and tetracycline groups.

There was a statistically significant reduction in the average number of episodes of lower respiratory illness per patient per year of observation for all drug regimens. There was also a concurrent reduction in the mean number of days of morbidity per patient per year but the figure for the penicillin group was not significantly less

TABLE 2. *Effect of treatment on clinical features appraised*

| Characteristics after treatment | Drug group | | | |
|---|---|---|---|---|
| | Placebo | Penicillin | Oleandomycin-penicillin | Tetracycline |
| Average sputum volume (ml/24 hr) | 210 | 180 | 120 | 90 |
| Sputum color | | | | |
| White | 72 | 78 | 115 | 101 |
| Yellow | 46 | 36 | 18 | 28 |
| Green | 32 | 30 | 6 | 20 |
| Episodes lower respiratory infections per patient per year | | | | |
| Total | 2.3 | 1.5 | 1.4 | 1.3 |
| Febrile only | 0.86 | 0.62 | 0.49 | 0.22 |
| Episodes upper respiratory infections per patient per year | 1.4 | 1.7 | 1.3 | 1.7 |
| Days (avg) lower respiratory infections per patient per year | | | | |
| Total | 9.2 | 6.0 | 4.9 | 4.8 |
| Febrile only | 2.2 | 1.9 | 0.8 | 0.3 |
| Additional treatment | | | | |
| Required for respiratory infections | 25 | 22 | 20 | 12 |
| Required for other reasons | 4 | 2 | 2 | 6 |
| Required for respiratory infections plus other reasons | 0 | 1 | 1 | 1 |
| Ratios | | | | |
| Febrile episodes LRI*/Total episodes LRI | 0.37 | 0.40 | 0.35 | 0.17 |
| Febrile days LRI/Total days LRI | 0.24 | 0.31 | 0.16 | 0.06 |
| Febrile days LRI/Febrile episodes LRI | 2.6 | 3.2 | 1.6 | 1.4 |
| Total days LRI/Total episodes LRI | 4.0 | 4.0 | 3.5 | 3.7 |

*Lower respiratory infection = LRI.

than that for the placebo group. When only the episodes and days of illness with fever are considered, it is seen that the averages for the tetracycline and oleandomycin-penicillin patients were significantly lower than those for the recipients of placebo and penicillin. The more favorable symptomatic course of the tetracycline group was reflected by a reduction in the number of times additional treatment was deemed necessary for respiratory tract infection.

As a test for the specificity of the reporting of morbidity and as a control for possible breaks or bias in the double-blind protocol, the upper respiratory infections were also recorded. It is interesting to see that the average number of upper respiratory illnesses was consistent among the groups.

That the effect of tetracycline was particularly marked on the degree of fever is best shown by the ratios tabulated. Thus, the ratio of the average number of febrile episodes to total episodes was lowered by tetracycline even though the average total days of morbidity was the same for each episode in all of the groups. Thus, the drugs reduced days of illness by reducing the number of episodes, but tetracycline markedly reduced the proportion of febrile

days to total days of illness and shortened the febrile course of the few febrile episodes which occurred. The oleandomycin-penicillin treated patients showed change in a similar direction but of lesser magnitude.

TABLE 3. *Frequency of isolation of microbial species from sputum (by 3-month intervals following treatment)*

| Number of organisms found* | Microbial species | | | | | | | | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | *H. influenzae* | | | | Pneumococcus | | | | *Staphylococcus* | | | | *Klebsiella-Aerobacter* | | | | *Pseudomonas* | | | | *Proteus* | | | |
| | 1† | 2 | 3 | 4 | 1 | 2 | 3 | 4 | 1 | 2 | 3 | 4 | 1 | 2 | 3 | 4 | 1 | 2 | 3 | 4 | 1 | 2 | 3 | 4 |
| None | 66 | 67 | 75 | 86 | 96 | 115 | 107 | 101 | 109 | 112 | 109 | 103 | 101 | 100 | 95 | 83 | 113 | 104 | 106 | 96 | 99 | 114 | 96 | 102 |
| Found intermittently | | | | | | | | | | | | | | | | | | | | | | | | |
| Few | 0 | 0 | 0 | 1 | 4 | 0 | 1 | 1 | 1 | 5 | 0 | 0 | 9 | 5 | 13 | 8 | 1 | 10 | 0 | 0 | 7 | 1 | 5 | 2 |
| Many | 30 | 28 | 22 | 12 | 11 | 3 | 2 | 2 | 4 | 1 | 0 | 1 | 2 | 4 | 0 | 3 | 1 | 3 | 3 | 4 | | | | |
| Found consistently | | | | | | | | | | | | | | | | | | | | | | | | |
| Few | 0 | 2 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 2 | 2 | 0 | 6 | 0 | 1 | 0 | 0 | 9 | 5 | 9 | 0 |
| Few and many, irregularly | 1 | 1 | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 2 | 0 | 2 | 0 | 1 | | | | |
| Many | 18 | 22 | 13 | 5 | 3 | 2 | 0 | 0 | 1 | 2 | 0 | 0 | 1 | 9 | 2 | 2 | 0 | 0 | 1 | 3 | | | | |
| Total periods with any organism | 48 | 53 | 35 | 18 | 19 | 5 | 3 | 3 | 6 | 8 | 1 | 1 | 14 | 20 | 15 | 21 | 2 | 16 | 4 | 8 | 16 | 6 | 14 | 2 |

*Definition of terms in Results section.

†Drug groups: placebo, (1); penicillin, (2); oleandomycin-penicillin, (3); and tetracycline, (4).

In Table 3 the comparison of the results of the bacteriological examinations of the sputum have been recorded. They have been tabulated by 3-month intervals so that the symptomatology which was tabulated from clinical material could be compared directly to the microbiological results. If an organism was found on some culture during the 3-month period and not on others, it was listed as intermittently positive. If it was present in all it was listed as being consistently found. When present, an estimate was made whether the given organism made up 50% or more of the total colonies on the plate. If so, it was listed as many, if not, it was listed as few. It is interesting to note that with *Haemophilus* there tended to be cyclical periods of relatively consistent isolation and, when present, it almost always predominated. Pneumococci also tended to predominate when present. The other genera were less consistent and *Proteus* could not be quantitated. Particular attention has been paid to the analysis of the data for six common aerobic genera. It was clearly demonstrated that the organisms most frequently isolated from all groups were strains of *H. influenzae* and the second most common were organisms in the *Klebsiella-Aerobacter* group. Only among the tetracycline group did organisms from the *Klebsiella-Aerobacter* group exceed strains of *H. influenzae*. The fact that none of the species was isolated from the majority of sputa was of interest.

The reduction in the frequency of isolation of *Haemophilus* strains, concurrent with tetracycline usage, was significant. Similarly, there was a significant reduction in the frequency with which pneumococci

could be isolated among all of the drug-treated patients. There was also a significant reduction in the strains of *Proteus* isolated from the patients who received tetracycline and penicillin. The only increase of significance was the frequency with which *Pseudomonas* strains have appeared in the penicillin group. It should be noted that there were proportionately more patients in this group who harbored the organism prior to therapy.

The relationship of the presence or absence of *Haemophilus* in the sputum in a given time interval has been related to the clinical symptomatology in that time period (Table 4). It is clear that, in the placebo group, the presence or absence of *Haemophilus* did not relate to either febrile or nonfebrile lower respiratory infections. It is also quite clear that each of the drug regimens was most effective in lowering both types of lower respiratory episodes in the periods of time when *Haemophilus* could not be isolated. However, the reduction in the average number of episodes, with and without fever in the drug groups for the periods during which *Haemophilus* was isolated, was not great, and, indeed, for penicillin the number of febrile episodes exceeded the number in the placebo group.

TABLE 4. *Isolation of Haemophilus influenzae and lower respiratory infections in each 3-month period after treatment began*

| Drug group and clinical status | Status of *Haemophilus** | | | |
|---|---|---|---|---|
| | Not isolated | Intermittant but predominant | Consistant and predominant | Total positive |
| Placebo | | | | |
| Avg episodes LRI† | 0.67 | 0.44 | 0.78 | 0.56 |
| Avg episodes LRI with fever | 0.25 | 0.16 | 0.23 | 0.21 |
| Episodes with fever | 35% | 36% | 28% | 32% |
| Penicillin | | | | |
| Avg episodes LRI | 0.36 | 0.37 | 0.57 | 0.47 |
| Avg episodes LRI with fever | 0.13 | 0.38 | 0.25 | 0.32 |
| Episodes with fever | 33% | 100% | 36% | 67% |
| Oleandomycin-penicillin | | | | |
| Avg episodes LRI | 0.27 | 0.35 | 0.45 | 0.39 |
| Avg episodes LRI with fever | 0.09 | 0.15 | 0.17 | 0.15 |
| Episodes with fever | 33% | 43% | 40% | 42% |
| Tetracycline | | | | |
| Avg episodes LRI | 0.36 | 0.63 | 0.25‡ | 0.55 |
| Avg episodes LRI with fever | 0.05 | 0.21 | 0.0 ‡ | 0.17 |
| Episodes with fever | 13% | 30% | -----‡ | 27% |
| All groups | | | | |
| Avg episodes LRI | 0.43 | 0.43 | 0.59 | 0.48 |
| Avg episodes LRI with fever | 0.12 | 0.22 | 0.20 | 0.22 |
| Episodes with fever | 25% | 50% | 35% | 43% |

**Haemophilus* isolation classified as: none isolated; isolated intermittently on several cultures during each tabulated 3-month period but, when present, it was predominant; and isolated consistently on all cultures in the period and predominant.

†Average total episodes lower respiratory infections per patient per 3 months for all appropriate 3-month periods.

‡Only four time periods. Insufficient data for analysis.

Therefore, the percentage of episodes of lower respiratory illness, which were associated with fever, was somewhat higher in the penicillin and oleandomycin-penicillin-treated patients when *Haemophilus* strains were isolated.

## Discussion

There have been repeated reports that continuous use of various antibiotics over a relatively wide dosage range will lower the morbidity of patients with chronic bronchial disease (Cherniack et al., 1959; Medical Research Council, 1957). In this study the same phenomenon has been demonstrated. A larger than usual dose of tetracycline was used to minimize the persistence of strains of *H. influenzae*, since we were particularly interested in evaluating their role in the chronic, as well as the acute, morbidity. The other drug regimens were chosen to try to achieve removal of the pneumococcus without broad coverage (penicillin) and broad coverage with little anti–*Haemophilus* activity (oleandomycin-penicillin). In this respect the study has been partially successful, since there was a preferential lowering of the frequency of *Haemophilus* isolation in the tetracycline-treated patients, although it was not as complete as was hoped for. Moreover, the oleandomycin-penicillin regimen may have been somewhat active in this regard.

As anticipated, we verified that use of the drugs was associated with a lowering of acute morbidity. The tetracycline regimen appeared to have the most favorable results, particularly with fever. The absence of fever may have accounted for the less frequent use of supplemental antibiotics. The more extensive reduction of febrile days and episodes raised the question of whether this could be a nonspecific antipyretic effect. However, the fact that the oleandomycin-penicillin group had the same effect, to a lesser degree, makes it more likely that the antibacterial effect is important. The major difference between oleandomycin-penicillin and tetracycline might be the more complete removal of *Haemophilus* strains. Even though the presence or absence of *Haemophilus* strains did not alter the symptomatology in the placebo group, their persistence seemed to minimize the effectiveness of penicillin and oleandomycin-penicillin. The number of periods in which *Haemophilus* was found in tetracycline-treated patients was so small that the results are too meager to interpret.

The favorable effect shown was the reduction of acute episodes of morbidity. Whether in the long run this effect, or even the consistent suppression of organisms, will favorably affect the chronic progressive damage has not been established. At this time there is no compelling reason to assume such a relationship.

## Literature Cited

Cherniack, N. S., K. L. Vosti, H. F. Dowling, M. H. Lepper, and G. G. Jackson. 1959. Long-term treatment of bronchiectasis and chronic bronchitis. A. M. A. Arch. Internal Med. 103: 345-353.

Medical Research Council. 1957. Prolonge antibiotic treatment of severe bronchiectasis. Brit. Med. J. 2:255-258.

# INTRAVENOUS SODIUM NITROFURANTOIN: A LIMITED STUDY[1]

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Oral nitrofurantoin is widely employed for the treatment of bacterial infections of the urinary tract. After ingestion the drug is readily absorbed and excreted largely in the urine. The resulting high drug levels in urine are inhibitory or bactericidal for a majority of bacteria commonly found in urinary tract infections (Kleeman, Hewitt, and Guze, 1960). With daily doses of 600 mg, urinary concentration of nitrofurantoin may reach 240 $\mu$g per ml, determined as antibacterial activity against test microorganisms. However, there is no antibacterial activity in serum or plasma, perhaps because the drug is firmly bound to blood protein (Richards et al., 1955; Jawetz, Hopper, and Smith, 1957). There appears to be general agreement that nitrofurantoin administered orally has no effect on bacteria in blood or tissues outside of the urinary tract. Partly for this reason, an intravenous dosage form was marketed some years ago, in the anticipation that infusion into the blood stream would lead to significant antibacterial concentrations in blood, tissue fluids, and organs. Polyethylene glycol 300 was employed in that preparation because of the relative insolubility of nitrofurantoin in water. In a careful study of 30 patients (McCabe, Jackson, and Grieble, 1959), it was found that the daily administration of 240 mg of nitrofurantoin in polyethylene glycol resulted in severe metabolic acidosis and nephropathy in seven individuals with two deaths. These damaging effects were attributed to glycol rather than nitrofurantoin, and that dosage form was withdrawn from the market.

Recently, the water-soluble sodium nitrofurantoin has been produced for intravenous administration. Whenever a new dosage form, or a slight modification of an established drug is developed, the pharmaceutical manufacturer desires the same extensive clinical trials that are usually accorded truly novel drugs, yet there is doubt whether large-scale trials are necessarily desirable in such cases. In the recent literature, there are many examples showing that very considerable investigative effort was expended to demonstrate minimal or questionable superiority of a slightly modified molecule over the parent drug. In the present study we intended to apply the opposite

[1]This paper has been submitted for publication in the *New England Journal of Medicine*.

approach: What could be learned from a very limited study on a carefully observed small number of patients, when dealing with a new dosage form of an established drug?

## Materials and Methods

*Drug.* Sodium nitrofurantoin (supplied by Eaton Laboratories) was dissolved in 5% dextrose in water. The total daily dose was administered either as one continuous slow intravenous infusion of 1,000 ml given in 12 to 14 hr or as two daily infusions of 500 ml each, given in two periods of 4 hr each.

*Patients.* Individuals admitted to the study either exhibited signs of acute pyelonephritis with positive urine cultures or had evidence of chronic urinary tract infection by history and repeated quantitative urine cultures. The latter were performed on freshly voided midstream specimens of urine by standardized techniques (Hopper, Jawetz, and Hinman, 1953). All patients were hospitalized and closely observed.

*Assay of nitrofurantoin levels.* Specimens of sterile blood and clean voided urine were collected from patients during the second half of infusions. Serum and urine samples were kept at -20 C until examined in groups. Serial twofold dilutions were prepared in tryptose phosphate broth and inoculated with a 24-hr culture of *Streptococcus* SS (kindly made available by H. Brainerd) diluted to give a pre-incubation concentration of $5 \times 10^3$ colony-forming units per ml. Under these circumstances, the assay *Streptococcus* SS was completely inhibited and usually killed by from 1.5 to 3.0 $\mu$g sodium nitrofurantoin per ml.

*Microbial susceptibility.* The susceptibility to nitrofurantoin of microorganisms isolated from patients' urine was estimated by conventional twofold tube dilution tests, employing a pre-incubation concentration of approximately $10^4$ bacteria per ml. For each microorganism the minimal inhibitory concentration (MIC) was defined as the smallest drug concentration preventing grossly visible growth during 24 hr of incubation. In addition, we estimated the ability of the drug to produce prolonged inhibition, or killing, of the entire inoculum by prolonged incubation and subculture into drug-free medium.

## Results

Of the 10 patients participating in this limited study, the first two had urinary tract infections incidental to their far-advanced neoplasms. Although the infection was promptly suppressed by intravenous sodium nitrofurantoin and they appeared to tolerate the drug,

they succumbed to the metastasized tumors within a few days and are not included in this report. Details pertaining to the other eight patients are presented in Table 1. The in vitro susceptibility of the infecting gram-negative bacteria ranged from 6 to more than 96 μg sodium nitrofurantoin per ml. Response to treatment was clearly related to microbial susceptibility: The only microorganism not suppressed by nitrofurantoin in the urine was the resistant strain of *Proteus* in case no. 7.

The daily dose of sodium nitrofurantoin ranged from 180 to 720 mg and the total amount of drug administered in 6 to 10 days ranged from 1,115 to 6,840 mg. The drug levels in the urine varied from 60 to 240 μg per ml in seven patients. In the eighth patient, a woman suffering from renal tubular acidosis (case no. 4), no biologically active nitrofurantoin was demonstrable in the urine and the bacterial population in her urine remained entirely unaffected. This patient had no biologically active drug in the blood and she exhibited no toxic effects with daily doses up to 540 mg of sodium nitrofurantoin. The fate of the drug in this patient is obscure.

Repeated attempts were made to demonstrate antibacterial activity in serum from six patients, drawn during the last quarter of intravenous infusions. In only one serum specimen was an activity found equivalent to 3 μg nitrofurantoin per ml.

The prevalent clinical response to treatment with intravenous sodium nitrofurantoin is illustrated by the following case summary.

*Case no. 5.* This 30-year-old housewife had suffered intermittently from dysuria and flank pain for five years. Repeated urethral dilatations had been performed during the past year for "mild urethral stenosis." In the course of her first pregnancy, seven months before admission, she developed frequency, dysuria, back pain, and fever. A urine culture revealed more than $10^7$ *Escherichia coli* per ml. She was treated with sulfonamide for two weeks with some improvement, but six weeks before delivery the symptoms recurred, and she took 400 mg nitrofurantoin daily until confinement. Post partum she was asymptomatic for several weeks and there was no bacteriuria. However, 10 days before admission she suddenly developed malaise and nausea, progressing to chills, fever, and back pain. The patient was placed on oral nitrofurantoin but initially obtained no relief and then was unable to take the drug because of nausea and vomiting. A urine culture revealed $2 \times 10^6$ *E. coli* per ml. She was admitted to the hospital for parenteral treatment.

Physical examination showed an acutely ill, flushed, young woman with a temperature of 39.5 C, pulse 100, blood pressure 130 systolic and 75 diastolic. Except for marked bilateral tenderness of costovertebral angles, the physical findings were noncontributory.

Laboratory examinations indicated a hematocrit of 42%, white blood count of 15,000 with polymorphonuclear leucocytes, 85%; lymphocytes, 10%; and monocytes, 5%. The serum creatine was 0.8 mg per

TABLE 1. *Treatment of patients with urinary tract infections*

| Patient | | | | Organisms in urine | | | Dose (iv) | | | Bacteriological response | Clinical course |
|---|---|---|---|---|---|---|---|---|---|---|---|
| No. | Sex | Age | Known duration of urinary tract infection | Type | No. of organisms per ml | MIC* | Total | Days† | Maximal urine level | | |
| | | *yr* | | | | *μg/ml* | *mg* | | *μg/ml* | *colonies/ml* | |
| 1 | F | 34 | 3 months | *E. coli* | $10^7$ | 24 | 1,115 | 6(B) | 100 | $<10^2$ in 24 hr | Became afebrile in 24 hr. Bacteruria recurred 6 days after stopping medication. |
| 2 | F | 18 | 2 days | *Proteus* (after cath) | $10^7$ | 96 | 2,160 | 6(A) | 240 | $<10^2$ in 24 hr | Asymptomatic throughout. No bacteriuria 3 months later. Cure? |
| 3 | F | 18 | 5 days | *E. coli* | $3 \times 10^6$ | 24 | 2,520 | 7(B) | 60 | $<10^3$ in 24 hr | Became afebrile in 24 hr. No bacteriuria 3 months later. Cure? |
| 4 | F | 59 | 39 yr | *E. coli* | $3 \times 10^6$ | 24 | 3,160 | 8(A) | <10 | $10^6$-$10^7$ throughout | Patient has renal tubular acidosis. No measurable amount of nitrofurantoin found in either urine or blood |
| 5 | F | 30 | 5 yr | *E. coli* | $2 \times 10^6$ | 6 | 3,780 | 10(A) | >240 | $<10^2$ in 24 hr | Became afebrile in 24 hr. Nitrofurantoin 400 mg p.o. daily kept bacteriuria below 1,000 colonies/ml for 3 months |
| 6 | M | 49 | 3 days | *E. coli* (after cath) | $10^6$ | 48 | 3,960 | 8(A) | 240 | $<10^2$ | Became afebrile in 24 hr asymptomatic maintained on nitrofurantoin p.o. |
| 7 | F | 18 | 15 yr | *Proteus* | $10^8$ | >96 | 5,400 | 10(A) | 240 | $10^4$-$10^5$ throughout | Asymptomatic throughout. Bacteriuria returned 10 days after iv medication |
| 8 | M | 39 | 2 wk | *Proteus* (after cath) | $10^7$ | 96 | 6,840 | 10(A) | 240 | $<10^2$ | Urinary tract infection controlled but patient died with dissem. nocardiosis |

*Minimal inhibitory concentration of nitrofurantoin in vitro for the organism.

†Single infusion, 12 hr per day (A) and two infusions, 4 hr each, at 12 hr intervals (B).

100 ml and the creatinine clearance was 123 ml per min. Cephalin flocculation and thymol turbidity tests were negative. The urine had a specific gravity of 1.004 (pH 5.5) and contained no sugar or protein. In the urinary sediment there were a few red and many white blood cells and bacteria. Urine culture yielded $10^5$ *E. coli* per ml. The organism was susceptible to several antibiotics and was inhibited by sodium nitrofurantoin 6 μg per ml.

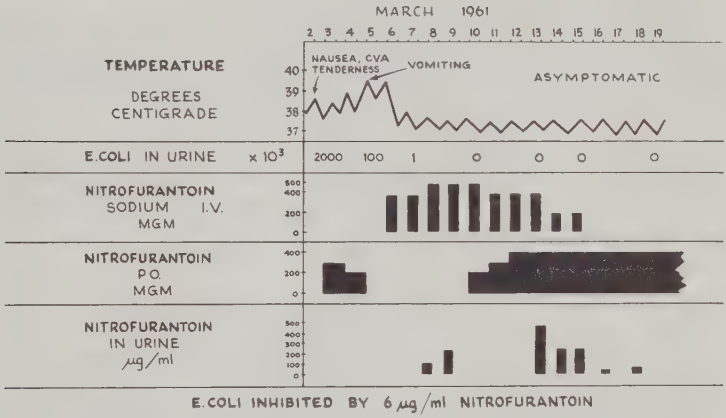
MARCH 1961
2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19
TEMPERATURE
DEGREES CENTIGRADE
NAUSEA, CVA TENDERNESS
VOMITING
ASYMPTOMATIC
E.COLI IN URINE x 10³
2000 100 1 0 0 0 0
NITROFURANTOIN SODIUM I.V. MGM
NITROFURANTOIN P.O. MGM
NITROFURANTOIN IN URINE μg/ml
E.COLI INHIBITED BY 6 μg/ml NITROFURANTOIN

FIG. 1. *Clinical course of patient no. 5, a 30-year-old housewife with recurring urinary tract infection for five years, treated with intravenous sodium nitrofurantoin.*

Treatment and course of this patient are depicted in Fig. 1. Within less than 12 hr after the start of intravenous nitrofurantoin, there was marked symptomatic improvement and defervescence and bacteriuria was no longer demonstrable. On the fifth day of treatment, oral nitrofurantoin was added and the dose of intravenous sodium nitrofurantoin gradually decreased. After 10 days of intravenous sodium nitrofurantoin, the patient was kept on oral nitrofurantoin, 400 mg daily. She remained free from symptoms and urine cultures were sterile for four months, up to the time of writing. While receiving intravenous nitrofurantoin, her drug levels in urine repeatedly reached 240 μg per ml whereas on oral nitrofurantoin they did not exceed 32 to 64 μg per ml.

*Comment.* This patient illustrates a reasonable clinical indication for intravenous sodium nitrofurantoin. An acutely ill person with pyelonephritis, unable to take oral medication, can be dramatically improved in a few hours, and the bacteriuria can be promptly and completely suppressed. When the patient improves, oral drug can be substituted.

Of the eight patients, six had a similar, satisfactory response with dramatic subsidence of symptoms (present in five patients) and

suppression of bacteriuria. Of these patients, two young women, with infection of only a few days' standing, showed no recurrence of clinical illness of bacteriuria in 3 months of follow-up, and, thus, may have been cured by the intravenous medication (cases no. 2 and 3).

Clinical failure was associated, in one patient, with a lack of nitrofurantoin excretion in urine (case no. 4) and with the presence of a resistant microorganism in another patient.

Nitrofurantoin has been associated with occasional gastrointestinal, hematological, and metabolic disturbances. We searched for evidence of such adverse reactions in patients receiving intravenous sodium nitrofurantoin. The infusion was generally well tolerated and induced nausea only if the rate of flow was too rapid. Thrombophlebitis was not encountered. There was no suggestion of bone marrow depression, hemolytic effects, or renal toxicity. A possible hepatotoxic effect (case no. 7) manifested itself by a transient rise in cephalin flocculation test results.

## Discussion

Within the limitations of the present study, some pertinent answers were obtained.

First, intravenous sodium nitrofurantoin was rapidly excreted. Antibacterial concentrations in urine were inhibitory for seven of eight strains of infecting microorganisms within a few hours after starting an infusion. With doses of 360 to 540 mg, infused over a 12- to 14-hr period, urine levels up to 240 $\mu$g nitrofurantoin per ml were achieved. These were two to four times higher than levels in the urine of the same patients on an oral dose of 400 mg nitrofurantoin.

Second, in only one of six patients' serum was any antibacterial activity demonstrable toward the end of an infusion of 540 or 720 mg sodium nitrofurantoin. In that one instance, the activity was equivalent to 3 $\mu$g nitrofurantoin per ml—the MIC of the most susceptible microorganism available to us. Thus, we found no evidence to support the belief that intravenous sodium nitrofurantoin would be active in systemic bacterial infections.

Third, with the sole activity of intravenous sodium nitrofurantoin thus established in the urinary tract, there evolves clearly one specific indication for its use: the acutely ill, nauseated, or vomiting patient with urinary tract infection. Many such individuals undoubtedly would also respond to other antimicrobial drugs given parenterally, but the prompt efficacy of intravenous sodium nitrofurantoin and its activity against a large majority of infecting microorganisms are points in favor of the new dosage form.

In spite of giving very large doses of the drug we encountered no serious toxic effects in this trial. There is need to investigate further the possibility of hepatotoxic effects suggested by positive cephalin flocculation tests seen in one patient who received up to 7 mg per lb per day instead of 3 mg per lb suggested by the manufacturer.

## Acknowledgment

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## Literature Cited

Hopper, J., E. Jawetz, and F. Hinman. 1953. Polymyxin B in chronic pyelonephritis: observations on the safety of the drug and on its influence on the renal infection. Am. J. Med. Sci. **225**: 402-409.

Jawetz, E., J. Hopper, and D. R. Smith. 1957. Nitrofurantoin in chronic urinary tract infection. A. M. A. Arch. Internal Med. **100**:549-557.

Kleeman, C. R., W. L. Hewitt, and L. B. Guze. 1960. Pyelonephritis. Medicine **39**:3-116.

McCabe, W., G. C. Jackson, and H. G. Grieble. 1959. Treatment of chronic pyelonephritis. A. M. A. Arch. Internal Med. **104**:710-719.

Richards, W. A., E. Riss, E. H. Kass, and M. Finland. 1955. Nitrofurantoin. Clincal and laboratory studies in urinary tract infections. A. M. A. Arch. Internal Med. **96**:437-450.

# TREATMENT OF PERSISTENT BACTERIURIA WITH A SIX-WEEK COURSE OF ANTIBIOTIC THERAPY

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Persistence or recurrence of bacteriuria following short courses of antimicrobial therapy is a phenomenon well known to clinicians treating infections of the urinary tract. This is particularly true in patients with long-standing chronic infections The paucity of cures in these patients may be related to factors indigenous to renal tissue, which interfere with eradication of microorganisms or promote microbial persistence. It has been postulated that infections of the urinary tract are somewhat analogous to bacterial endocarditis in which brief courses of antibiotic therapy sometimes fail to eliminate the causative pathogens, resulting in relapses which, in turn, are eliminated merely by continuation of the same antibiotic regimen for a more prolonged period.

The present study, which is still in progress, was undertaken to determine whether prolonged therapy of urinary tract infections would result in a diminution of recurrences and in more permanent cures in chronic bacteriuria.

## Materials and Methods

*Selection of patients for study.* During a 12-month period, 122 ambulatory patients were referred to the "Pyelonephritis Clinic" at the King County Hospital because of suspected urinary tract infection. Of these, 64 had bacterial counts greater than 100,000 per ml of urine on at least one clean-voided specimen. To be included in the study each patient had to meet the following criteria: (i) successive quantitative urine cultures obtained at weekly intervals showing bacteriuria greater than 100,000 per ml with a gram-negative bacillus; (ii) isolation of the same microorganism(s) on these two consecutive cultures; (iii) absence of instrumentation or catheterization during treatment or observation period; (iv) administration of no

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antimicrobials other than the "study drug" during the period of observation and treatment; and (v) follow-up bacteriological data for at least 6 weeks after cessation of therapy.

To date, 27 patients have fulfilled these criteria and form the basis for this report. Prior to therapy, 24 of 27 had "complicated" urinary infections characterized by surgery, instrumentation, or calculi. All had received one or more short courses of antibiotics or sulfonamides in the past and had at least 2 months (average, 7 months) of documented gram-negative bacteriuria prior to treatment. All had had urologic work-ups, and obstructive uropathy had been eliminated wherever possible. Their ages ranged from 21 to 87, with the majority in the geriatric group. There were 14 males and 13 females. In most patients infections were asymptomatic at the onset of therapy, although symptoms referable to urinary infections had been present in the past.

*Selection of antibiotics.* Antibiotics were chosen on the basis of in vitro sensitivity tests. Patients whose organisms were sensitive to demethylchlortetracycline (DMCT) were given this agent in dosage of 600 mg/day. Pathogens, resistant to DMCT but sensitive to chloramphenicol, were treated with the latter in dosage of 2 g/day, and cycloserine (1 g/day) was administered to patients whose organisms were resistant to both DMCT and chloramphenicol. To permit objective evaluation of symptoms and urinary sediments, each of the first 17 patients was assigned to a drug or placebo group at random, and after 6 weeks the schedule was reversed. The succeeding ten patients were given only the antimicrobial. In all, 12 patients received DMCT, 6 were treated with chloramphenicol, and 9 with cycloserine. In four of the cycloserine-treated group, dosage was reduced to 500 mg/day because of toxic manifestations.

*Laboratory and clinical studies.* Patients were followed at biweekly intervals and clean-voided urine specimens obtained for quantitative culture. Antimicrobial sensitivities were determined by the tube-dilution and single-disc techniques. Serological typing was performed on *Escherichia coli* isolates, employing the scheme of Edwards and Ewing (1955).

## Results

*Bacteriuria.* The effect of long-term antimicrobial therapy on bacteriuria is summarized in Table 1. Patients responded in four distinct patterns. (i) Bacteria disappeared from the urine during therapy and patients remained free of infection. (ii) Pathogens present at the onset of therapy persisted during treatment or recurred following cessation of treatment. (iii) Organisms present at onset of treatment were replaced by other microbial species during or shortly after treatment period. (iv) One patient had spontaneous reduction of bacteriuria during the pretreatment observation period.

TABLE 1. *Results of long-term antibiotic therapy*

| Drug | Cases* | No bacteruria | Persistent initial pathogen | Replacement initial pathogen | Spontaneous reduction |
|---|---|---|---|---|---|
| | *no.* | | | | |
| DMCT | 12 (6) | 1 | 3 | 7 | 1 |
| Chloramphenicol | 6 (4) | 2 | 2 | 2 | 0 |
| Cycloserine | 9 (1) | 1 | 8 | 0 | 0 |

*Figure in parentheses refers to patients who were abacteriuric on therapy.

*Disappearance of bacteriuria.* Four patients remained free of infection for at least 6 weeks following therapy. The course of one patient, a 77-year-old man, who had persistant bacteriuria 9 months following transurethral prostatic resection is illustrated in Fig. 1. *E. coli* disappeared from the urine shortly after therapy was instituted; the urine has remained free of bacteria during the ensuing 8 months. Two other patients in this group also had persistent infection following prostatic surgery; the fourth had had repeated catheterization during pregnancy. In general, these patients had fewer factors favoring persistence of infection than those with refractory bacteriuria. Cultures became sterile shortly after institution of therapy in all four, and we did not observe a single patient

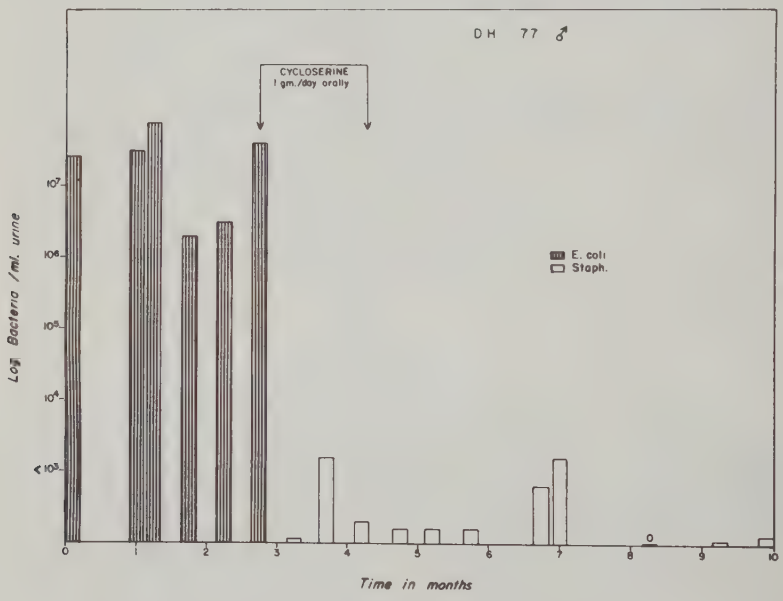


FIG. 1. *Disappearance of causative pathogen with cycloserine.*

in any group with bacteriuria 2 weeks after beginning of treatment who became abacteriuric 4 to 6 weeks after starting therapy.

*Persistence of initial pathogen.* In three patients bacteriuria was suppressed with antimicrobials, only to return after treatment was discontinued. An example of this is given in Fig. 2, a 21-year-old woman with a normal collecting system, who had had symptomatic urinary tract infection since pregnancy, 9 months previously. *E. coli*, O group 6, was isolated before treatment, bacterial counts fell to insignificant levels on chloramphenicol, but a strain of the same group was cultured after the drug was stopped. In this instance, serological typing permitted more precise identification of the causative pathogen.

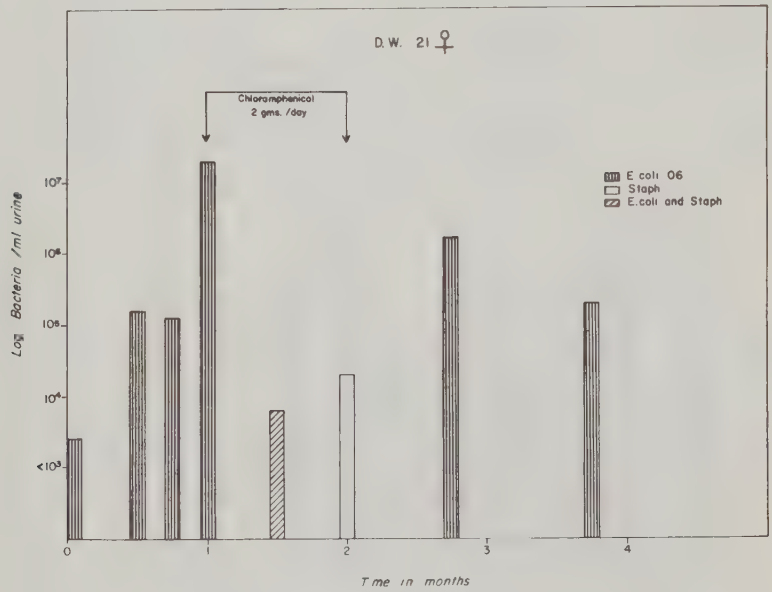


FIG. 2. *Recurrence of E. coli 06 following discontinuation of treatment with chloramphenicol.*

In the majority of patients in whom the primary pathogen persisted in the urine, cultures never became sterile. *Proteus*, *Pseudomonas*, and a combination of organisms were the offending pathogens (Table 2). An example of this type of response is provided in Fig. 3, depicting the course of a 62-year-old woman with diabetes mellitus, diabetic neuropathy and a cord bladder, and a history of multiple urinary instrumentations. She had been parasitized by *Pseudomonas* for more than a year, and a 6-week course of cycloserine failed to eradicate the organism.

TABLE 2. *Antimicrobials used compared with specific pathogens*

| Initial pathogen | Number used | DMCT | Chloramphenicol | Cycloserine |
|---|---|---|---|---|
| *E. coli* | 12 | 7 | 4 | 1 |
| *P. mirabilis* | 4 | 0 | 1 | 3 |
| *Klebsiella* | 4 | 4 | 0 | 0 |
| *Pseudomonas* | 4 | 0 | 0 | 4 |
| Pathogenic *Serratia* | 1 | 0 | 1 | 0 |
| Mixed | 2 | 1 | 0 | 1 |

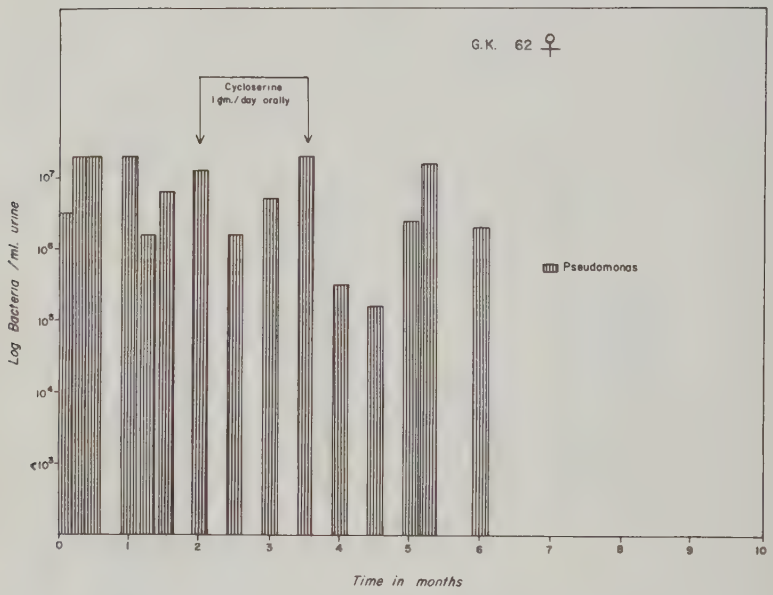


FIG. 3. *Persistence of Pseudomonas bacteriuria during a 6-week course of cycloserine.*

*Replacement of initial pathogen by another bacterial species or different serotype of same species.* In nine patients, the organism cultured from the urine prior to therapy was replaced by a different strain. In six of these nine the superinfection did not appear until treatment had been completed, and in three others the initial pathogen was supplanted during the administration of antibiotics. A typical example of this type of response is summarized in Fig. 4, describing an 84-year-old woman with a cystocoele and long-standing

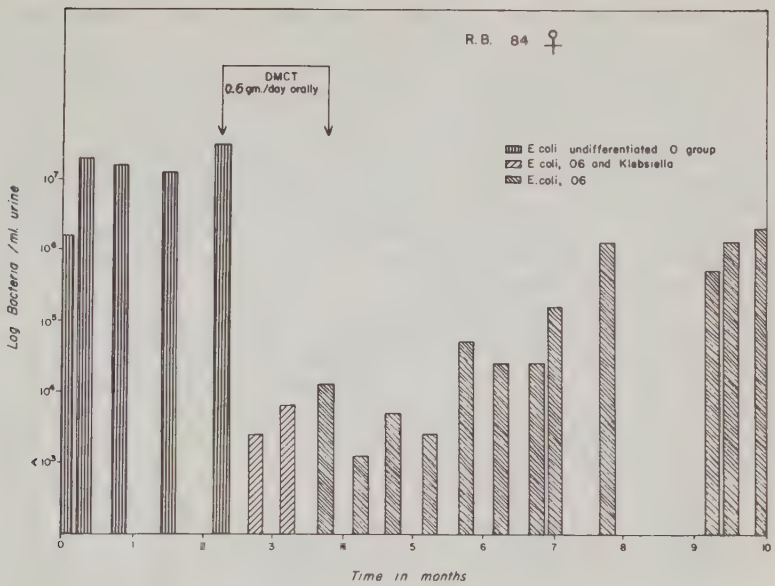


FIG. 4. *Superinfection with a different pathogen following a course of antimicrobial therapy.*

bacteriuria. *E. coli* of an undifferentiated O group was cultured prior to treatment. During treatment with antibiotics, the number of bacteria in the urine diminished but the superinfecting organism, *E. coli* O-6, made its appearance. Free from the bacteriostatic effect of antibiotics, this organism became established in the urinary tract and has persisted for 8 months following completion of therapy. It is of interest that the initial strain of *E. coli* was sensitive to DMCT, but its successor was markedly resistant to this agent. Of the nine initial pathogens, seven were strains of *E. coli* and the other two were *Klebsiella.* In six instances these were replaced by *Proteus mirabilis* and in one each by another strain of *E. coli, Klebsiella,* and a combination of paracolon and *P. rettgeri.* In general, the secondary organisms were far more resistant than their predecessors.

*Relationship of therapeutic response to antimicrobials administered.* Table 2 indicates the strains isolated and the antibiotics employed. All but one of 16 patients harboring *E. coli* and *Klebsiella* were treated with DMCT or chloramphenicol. On the other hand, by virtue of their resistance to these two agents, seven of eight strains of *Proteus* and *Pseudomonas* were treated with cycloserine. Since patients with more resistant pathogens were treated with cycloserine, this agent was put at a disadvantage with respect to the two others.

On the basis of these data, no fair comparison between different antimicrobials can be made.

From a prognostic point of view, the results of therapy became clearer when antibiotics were eliminated from consideration and the outcome of infection was correlated with the causative microorganisms (Table 3). The only patients who became abacteriuric during therapy were infected with *E. coli*, *Klebsiella*, and pathogenic *Serratia*. This was also the group most prone to superinfections with other organisms. In contrast, patients with *Proteus*, *Pseudomonas*, and mixed infections retained the initial pathogen and did not become abacteriuric during therapy.

TABLE 3. *Response of different microbial genera to antibiotics*

| Organism | Cases* | No bacteriuria | Persistence initial pathogen | Replacement initial pathogen | Spontaneous reduction |
|---|---|---|---|---|---|
| | *no.* | | | | |
| *E. coli* | 12 (7) | 2 | 2 | 7 | 1 |
| *P. mirabilis* | 4 (0) | 0 | 4 | 0 | 0 |
| *Klebsiella* | 4 (3) | 1 | 1 | 2 | 0 |
| *Pseudomonas* | 4 (0) | 0 | 4 | 0 | 0 |
| Pathogenic *Serratia* | 1 (1) | 1 | 0 | 0 | 0 |
| Mixed | 2 (0) | 0 | 2 | 0 | 0 |

*Figure in parentheses refers to patients who were abacteriuric on therapy.

*Relation of pyuria to bacteriuria.* Of 17 patients, 15 had more than 50 white cells per high-power field in the centrifuged specimen

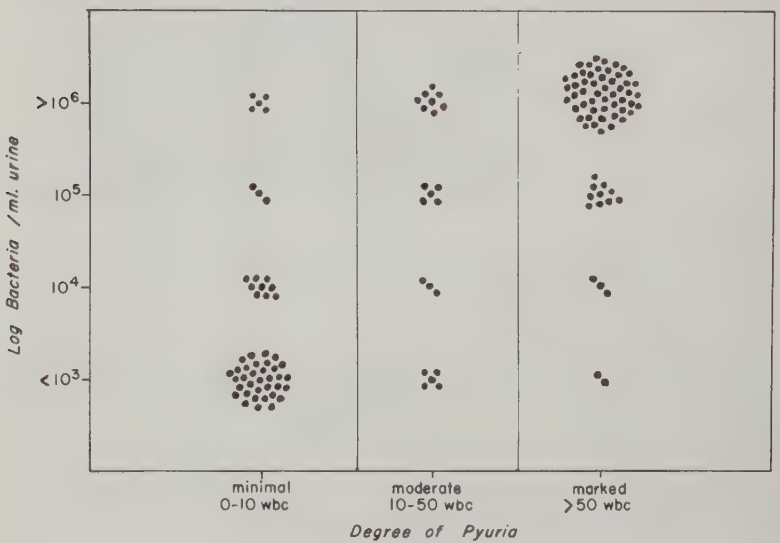


FIG. 5. *Relation of pyuria to bacteriuria.*

of the urinary sediment; all of these had more than $10^5$ bacteria per ml of urine. There was a direct correlation between the magnitude of bacteriuria and the number of cells in the sediment (Fig. 5). As bacteriuria decreased, there was diminution of pyuria in almost all instances. Inspection of the unstained sediments from freshly voided urine also demonstrated good correlation between the number of bacteria seen and the quantitative bacterial count. This has recently been reported by Kunin (1961).

*Symptomatic response.* An attempt was made to relate the patients' symptomatic response to bacteriuria employing a double-blind technique. The most common complaint was dysuria, which was present in most symptomatic patients. There was no relation whatever between therapeutic response and symptoms. Estimates by the authors as to whether patients were receiving drugs or placebo were correct in only a few instances.

*Toxic manifestations.* Toxicity was fairly common in patients treated with 1.0 g of cycloserine daily and consisted primarily of dizziness and ataxia. A dosage of 500 mg/day was tolerated with no untoward effects. One patient had transient anemia and leukopenia after 4 weeks of therapy with chloramphenicol; the findings in the bone marrow were consistent with toxic depression. As many patients complained of nausea, diarrhea, and nonspecific epigastric distress while on placebo as on DMCT.

## Discussion

These results confirm the well-known refractoriness of patients with chronic bacteriuria to antimicrobial therapy, and the hope that infection could be eradicated by prolonging the course of therapy was not realized. In fact, the results are comparable to those obtained by Grieble and Jackson (1958), with protracted courses of sulfamethoxypyridazine. These authors obtained a cure rate of 21%, in patients with chronic bacteriuria, and only infections caused by *E. coli* were eradicated.

Our data should be interpreted, however, in the light of the patients studied. Many may have had persistent obstructive uropathy, but could not be helped by further surgical intervention. In four instances, chronic bacteriuria was abolished with this intensive regimen, and in one of these patients, a young woman, bacteriological cure might well have forestalled the late development of chronic pyelonephritis.

The consistent findings that organisms disappear from the urine, if they disappear at all, during the first 2 weeks of therapy is of considerable practical importance, since it permits a rational approach to the use of antibiotics which are expensive and potentially hazardous. Unless patients become abacteriuric during the first 2 weeks of therapy, there is no reason to continue treatment, since only

those whose cultures were sterile after 2 weeks ultimately remained free of infection. Not answered as yet is the question whether the patients, whose urine became sterile during therapy and who remained abacteriuric, would have remained free of infection had they been treated for only 2 weeks. This problem is being investigated.

No statement can be made about the relative efficacy of antibiotics employed in the study. As has been mentioned, DMCT and chloramphenicol were used against relatively sensitive organisms compared to cycloserine, making any comparison invalid. It is possible that use of cycloserine, instead of DMCT or chloramphenicol, against *E. coli* and *Klebsiella* would have resulted in a similar rate of cure. There is little question that patients with infections due to *P. mirabilis* would have done better with large doses of penicillin (Hook and Petersdorf, 1960) and that *Pseudomonas* might have been eradicated more efficiently with colistin. However, these agents do not lend themselves to protracted therapy in ambulatory patients. Furthermore, even if *Proteus* and *Pseudomonas* had been eradicated, they might well have been replaced by other flora. It is our opinion at present that the antimicrobial used is not nearly as important in determining the outcome of infection as the relationship between host and parasite, about which much remains to be learned.

Finally, observation of many patients with chronic bacteriuria has made us aware that there are spontaneous fluctuations in both the number of organisms and bacterial species. For example, 18 patients referred for study became abacteriuric while under observation to document the presence of urinary infection. This must be taken into consideration in attempting to assess the effectiveness of antibiotics in these infections.

Since the presence of large numbers of leukocytes in the urinary sediment was almost always indicative of significant bacteriuria, examination of the unstained sediment for these cells and bacteria might eliminate the need for quantitative urine cultures in patients *known to have chronic bacteriuria*. However, it is important to remember that urinary infections may exist in the absence of pyuria, and examination of the unstained sediment should never be substituted for quantitative culture unless the presence of infection has been established by several cultures of the urine.

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## Literature Cited

Edwards, P., and W. H. Ewing. 1955. Identification of Enterobacteriaceae. Burgess Publishing Co. Minneapolis.

Grieble, H. G., and G. G. Jackson. 1958. Prolonged treatment of urinary-tract infections with sulfamethoxypyridazine. New Engl. J. Med. **258**:1-7.

Hook, E. W., and R. G. Petersdorf. 1960. *In vitro* and *in vivo* susceptibility of *Proteus* species to the action of certain antimicrobial drugs. Bull. Johns Hopkins Hosp. **107**:337-348.

Kunin, C. M. 1961. The quantitative significance of bacteria visualized in the unstained urinary sediment. New Engl. J. Med. **265**:589-590.

# SUSCEPTIBILITY OF GONOCOCCI ISOLATED PRIOR TO 1940 TO PENICILLIN AND OTHER ANTIMICROBIAL AGENTS

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The decrease in the susceptibility of *Neisseria gonorrhoeae* to penicillin has been demonstrated by the results of workers in various parts of the world. This increased tolerance of the *Gonococcus* to penicillin is of some concern, since it has been correlated with the increase in treatment failure rates (Cradock-Watson, Shooter, and Nicol, 1958; Curtis and Wilkinson, 1958; Reyn, Korner, and Bentzon, 1958; Thayer et al., 1961). One of the most recent publications (Fairbrother et al., 1961) includes an excellent summary of penicillin susceptibility surveys which had been reported from 1945 through 1960. This survey illustrates that, prior to 1949, no strains had been reported which were resistant to 0.06 unit (0.036 $\mu$g) of penicillin per ml; since 1949, strains resistant to this concentration have appeared with increasing frequency.

The susceptibility pattern of gonococci to penicillin prior to 1940 has not been reported. One of the major surveys on this subject (Reyn et al., 1958) included strains which had been isolated in 1944 and preserved in the lyophilized condition. These were compared with strains isolated in 1957. About 25% of the 1957 strains needed more than 0.135 unit (0.081 $\mu$g) per ml penicillin for complete inhibition in vitro. This was about 20 times as much penicillin as was required for most of the 1944 isolates.

We have been fortunate in obtaining from Sara E. Branham of the National Institutes of Health 15 strains of gonococci which were isolated and lyophilized prior to 1940 (1935 to 1939). In this investigation, these strains were tested for their susceptibility to penicillin G as well as 12 other antimicrobial agents.

These pre-1940 strains provided an opportunity to establish a base line of in vitro drug susceptibility of gonococci, prior to the era of antibiotic therapy. For comparative purposes, 15 strains of

recently isolated gonococci were included in this study and tested for their susceptibility to the same agents.

## Materials and Methods

*Cultures.* The strains of *N. gonorrhoeae* were divided into two groups, those isolated between 1935 to 1939, and those isolated between 1957 to 1961 (Table 1). They were maintained on cystine Trypticase agar deeps and transferred at weekly intervals. Strains

TABLE 1. *Comparison of penicillin susceptibility of strains of Neisseria gonorrhoeae isolated prior to 1940 to strains isolated from 1957 thru 1961*

| Strains isolated prior to 1940 | Minimal inhibitory concentration* | | | | | |
|---|---|---|---|---|---|---|
| | Penicillin G | | Phenoxyethyl-penicillin | | Dimethoxyphenyl-penicillin | |
| | Tube | Plate | Tube | Plate | Tube | Plate |
| | *units/ml* | | *μg/ml* | | *μg/ml* | |
| G1 | 0.015 | 0.015 | 0.03 | 0.03 | 0.03 | 0.03 |
| G2 | 0.015 | 0.03 | 0.06 | 0.125 | 0.06 | 0.06 |
| G3 | 0.015 | 0.03 | 0.06 | 0.125 | 0.03 | 0.06 |
| G4 | 0.015 | 0.03 | 0.03 | 0.06 | 0.03 | 0.03 |
| G5 | 0.008 | 0.03 | 0.125 | 0.25 | 0.125 | 0.25 |
| G6 | 0.008 | 0.06 | 0.06 | 0.25 | 0.06 | 0.125 |
| G7 | 0.015 | 0.03 | 0.06 | 0.125 | 0.03 | 0.06 |
| G8 | 0.008 | 0.03 | 0.125 | 0.125 | 0.03 | 0.06 |
| G9 | 0.015 | 0.03 | 0.06 | 0.125 | 0.06 | 0.06 |
| G10 | 0.015 | 0.03 | 0.125 | 0.125 | 0.06 | 0.06 |
| G12 | 0.015 | 0.03 | 0.03 | 0.125 | 0.03 | 0.06 |
| G15 | 0.015 | 0.03 | 0.125 | 0.125 | 0.06 | 0.06 |
| G17 | 0.008 | – | – | – | – | – |
| G20 | 0.015 | 0.03 | 0.06 | 0.125 | 0.06 | 0.06 |
| G24 | 0.015 | 0.06 | 0.06 | 0.25 | 0.06 | 0.125 |
| Average | 0.013 | 0.033 | 0.072 | 0.140 | 0.052 | 0.079 |
| Strains isolated from 1957 through 1961 | | | | | | |
| 26830 | 0.5 | 1.0 | 2.0 | 2.0 | 0.5 | 0.5 |
| 27438 | 0.06 | 0.25 | 0.5 | 1.0 | 0.5 | 0.5 |
| 29275 | 0.03 | 0.03 | 0.03 | 0.06 | 0.015 | 0.03 |
| 33250 | 0.25 | 0.50 | 1.0 | 2.0 | 0.5 | 1.0 |
| MSDH | 0.015 | 0.03 | 0.06 | 0.06 | 0.03 | 0.03 |
| RD-1 | 0.008 | 0.008 | 0.03 | 0.03 | 0.015 | 0.015 |
| F-18 | 0.03 | 0.06 | 0.125 | 0.25 | 0.25 | 0.25 |
| F-19 | 0.25 | 0.25 | 0.06 | 0.06 | 0.25 | 0.50 |
| F-21 | 0.25 | 0.25 | 0.125 | 0.125 | 0.25 | 0.50 |
| F-30 | 0.03 | 0.06 | 0.03 | 0.06 | 0.06 | 0.06 |
| F-31 | 0.06 | 0.06 | 0.125 | 0.125 | 0.03 | 0.06 |
| CH-1 | 0.03 | 0.03 | 0.004 | 0.008 | 0.008 | 0.008 |
| CH-2 | 0.06 | 0.06 | 0.125 | 0.125 | 0.03 | 0.06 |
| CH-5 | 0.25 | 0.50 | 0.25 | 0.50 | 0.25 | 0.50 |
| CH-6 | 0.25 | 0.50 | 0.125 | 0.25 | 0.25 | 0.50 |
| Average | 0.138 | 0.239 | 0.306 | 0.444 | 0.196 | 0.301 |

*MIC's determined by tube dilution and plate dilution techniques, respectively.

RD-1, F-18, F-19, F-21, F-30, and F-31 required the addition of 1% (v/v) supplement B (Difco).

*Antimicrobial agents.* The drugs used in this study included penicillin G, phenoxyethylpenicillin, dimethoxyphenylpenicillin, chlortetracycline, oxytetracycline, tetracycline, chloramphenicol, oleandomycin, oleandomycin-tetracycline, bacitracin, dihydrostreptomycin, polymyxin B, and sulfisoxazole. All drugs were received as dry crystalline material from which stock solutions were prepared using sterile distilled water. The stock solutions were stored at -20 C and used within 2 weeks of preparation.

*Susceptibility testing methods.* The minimal inhibitory concentration (MIC) of each antimicrobial agent for each strain was determined by the tube dilution technique. In addition, a plate dilution method was performed with penicillin G, phenoxyethylpenicillin, and dimethoxyphenylpenicillin.

*Tube dilution method.* The medium used in the tube dilution technique was of the following composition in g per liter: Trypticase (Baltimore Biological Laboratory, Inc.), 20; cystine, 0.5; sodium sulfite, 0.5; sodium chloride, 10; and agar, 0.7. Several strains, referred to previously, required the addition of 1% supplement B (Difco). The pH was adjusted to 7.4 to 7.5 and the medium sterilized in 300-ml amounts by autoclaving at 121 C for 20 min. The medium was dispensed aseptically in 4-ml amounts into sterile test tubes (15 × 125 mm). An appropriate dilution of the drug was added to the first two tubes of a series from which twofold serial dilutions were made.

Inocula were prepared from growth of the gonococci in biphasic culture (Gerhardt and Heden, 1960) using media of the composition described above except that the base layer was solidified by the addition of 2% agar. These flasks were inoculated from cystine Trypticase agar deeps and incubated 48 hr at 37 C. Growth in the liquid phase was adjusted to an optical density of 0.05 at 625 mμ using a Bausch and Lomb "Spectronic 20" colorimeter. This suspension was further diluted 1:2 and one drop of the resulting suspension, delivered from a 1.0-ml serological pipette, served as inoculum for each tube. The tubes were incubated 48 hr at 37 C. The MIC of a drug was recorded as the lowest concentration that inhibited visible growth.

*Plate dilution method.* Determination of the MIC of each drug for the *Gonococcus* strains was performed, using essentially the procedure described by Thayer, Garson, and Deacon (1960). This technique consisted of incorporating graded amounts of the drug in a chocolate agar medium followed by streak inoculation with standardized suspensions of the gonococci. The plates were incubated for 48 hr at 37 C in a candle jar and then observed for growth. The MIC was taken as the lowest concentration of the drug that inhibited visible growth.

## Results and Discussion

The susceptibility of *N. gonorrhoeae* to penicillin G, phenoxyethylpenicillin, and dimethoxyphenylpenicillin, as determined by the tube and plate dilution techniques, is shown in Table 1. In general, the MIC's obtained by the plate dilution method were slightly higher, frequently twice the value obtained by the tube method. In several instances, the values by either method were identical for a given culture.

*Penicillins.* From the tube dilution data, the average MIC of penicillin G for the pre-1940 strains was 0.013 unit/ml, and the recent isolates had an average MIC of 0.138 unit/ml, approximately a 10-fold difference. The difference by the plate method was approximately sevenfold. Recently isolated strains, with MIC's as low or lower than these pre-1940 strains, have been reported (Cradock-Watson et al., 1958; Curtis and Wilkinson, 1958; Hirsch and Finland, 1960; Odegaard, 1960) and may represent gonococci with no previous exposure to penicillin. The fact that none of the pre-1940 strains in this study had MIC's above 0.06 unit/ml, and the recently isolated strains exhibited MIC's as high as 1.0 unit/ml, is indicative of the transition that has occurred in terms of the susceptibility of *Gonococcus* to penicillin.

With phenoxyethylpenicillin, the pre-1940 strains had average MIC's of 0.072 and 0.140 $\mu$g/ml by the tube and plate methods, respectively. By the tube method, the recent isolates were approximately four times as resistant and by the plate method approximately three times as resistant.

Slightly increased resistance was also seen with dimethoxyphenylpenicillin. The average MIC's for the pre-1940 strains were 0.052 and 0.079 $\mu$g/ml by the tube and plate methods, respectively, and the recent isolates had average MIC's of 0.196 and 0.301 $\mu$g/ml by the two methods. Here, the difference is about the same as for phenoxyethylpenicillin, approximately a fourfold difference in both the tube and plate methods.

The susceptibility of the gonococci to other antimicrobial agents, as determined by the tube dilution technique, is shown in Table 2. The following observations are apparent from an examination of these data.

*Tetracyclines.* The three tetracyclines were, on the average, approximately two to three times more effective on the recent isolates than on the pre-1940 strains. This is in agreement with the findings of Hirsch and Finland (1960), who reported that strains of gonococci isolated in 1959 were slightly more susceptible to tetracyclines than were strains isolated in 1954. However, Reyn et al. (1958) reported no change in the susceptibility of gonococci to tetracyclines between 1944 and 1957. In the present study, oxytetracycline was slightly less effective with most strains than tetracycline, but slightly more effective than chlortetracycline.

TABLE 2. *Comparison of drug susceptibility of strains of Neisseria gonorrhoeae isolated prior to 1940 to strains isolated from 1957 thru 1961*

| Strains isolated prior to 1940 | Minimal inhibitory concentration of various antimicrobial agents | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | Chlortetracycline | Oxytetracycline | Tetracycline | Chloramphenicol | Oleandomycin | Bacitracin | Oleandomycin + tetracycline | Polymyxin B | Sulfisoxazole | Dihydrostreptomycin |
| | *μg/ml* | *μg/ml* | *μg/ml* | *μg/ml* | *μg/ml* | *units/ml* | *μg/ml* | *units/ml* | *μg/ml* | *μg/ml* |
| G1 | 0.6 | 10.0 | 2.5 | 0.6 | 0.6 | 0.3 | 0.6 | >160.0 | 5.0 | 0.3 |
| G2 | 5.0 | 5.0 | 1.25 | 0.6 | 1.25 | 0.6 | 2.5 | 40.0 | 5.0 | 0.6 |
| G3 | 0.6 | 5.0 | 2.5 | 0.6 | 1.25 | 0.6 | 1.25 | 10.0 | 5.0 | 0.3 |
| G4 | 0.6 | 10.0 | 0.6 | 0.6 | 0.15 | 0.08 | 0.3 | 80.0 | 2.5 | 0.3 |
| G5 | 1.25 | 10.0 | 5.0 | 0.6 | 1.25 | 0.15 | 1.25 | 5.0 | 160.0 | 0.15 |
| G6 | 0.6 | 1.25 | 2.5 | 0.3 | 0.3 | 0.15 | 0.6 | >160.0 | 10.0 | 0.08 |
| G7 | 5.0 | 5.0 | 2.5 | 1.25 | 0.3 | 0.3 | 1.25 | 10.0 | 10.0 | 0.08 |
| G8 | 10.0 | 2.5 | 2.5 | 0.6 | 0.6 | 0.3 | 1.25 | 40.0 | 5.0 | 0.3 |
| G9 | 20.0 | 5.0 | 5.0 | 0.6 | 1.25 | 0.3 | 1.25 | >160.0 | 5.0 | 0.15 |
| G10 | 10.0 | 2.5 | 2.5 | 0.6 | 2.5 | 0.6 | 2.5 | 20.0 | 10.0 | 0.15 |
| G12 | 5.0 | 2.5 | 0.6 | 0.6 | 0.3 | 0.08 | 0.6 | 40.0 | 5.0 | 0.6 |
| G15 | 10.0 | 10.0 | 5.0 | 0.6 | 1.25 | 0.6 | 2.5 | >160.0 | >160.0 | 0.3 |
| G17 | 20.0 | – | – | 0.6 | 2.5 | – | 2.5 | – | – | – |
| G20 | 2.5 | 1.25 | 1.25 | 0.6 | 0.3 | 0.6 | 2.5 | 40.0 | 5.0 | 0.6 |
| G24 | 2.5 | 5.0 | 2.5 | 0.6 | 0.6 | 1.25 | 1.25 | 40.0 | 10.0 | 0.04 |
| Average | 6.24 | 5.36 | 2.59 | 0.62 | 0.96 | 0.42 | 1.47 | | | 0.28 |
| Strains isolated from 1957 through 1961 | | | | | | | | | | |
| 26830 | 20.0 | 5.0 | 5.0 | 2.5 | 1.25 | 1.25 | 5.0 | 20.0 | 20.0 | 1.25 |
| 27438 | 2.5 | 0.6 | 2.5 | 0.6 | 0.3 | 0.15 | 0.6 | 160.0 | >160.0 | 0.3 |
| 29275 | 5.0 | 2.5 | 2.5 | 0.3 | 0.6 | 0.15 | 0.6 | >160.0 | 20.0 | 0.15 |
| 33250 | 5.0 | 2.5 | 2.5 | 0.3 | 0.3 | 0.08 | 0.6 | 80.0 | 10.0 | 10.0 |
| MSDH | 5.0 | 5.0 | 1.25 | 0.6 | 0.3 | 0.3 | 0.6 | 40.0 | 40.0 | 0.08 |
| RD-1 | 0.3 | 0.3 | 0.3 | 0.3 | 0.3 | 0.15 | 0.15 | >160.0 | 80.0 | 5.0 |
| F-18 | 0.3 | 0.3 | 0.3 | 0.3 | 0.6 | 2.5 | 0.15 | >160.0 | 20.0 | 5.0 |
| F-19 | 0.3 | 0.6 | 0.15 | 0.6 | 0.15 | 0.6 | 0.3 | >160.0 | 20.0 | 2.5 |
| F-21 | 0.6 | 0.6 | 0.3 | 0.3 | 0.15 | 0.3 | 0.3 | >160.0 | 10.0 | 10.0 |
| F-30 | 0.6 | 0.6 | 0.3 | 0.3 | 1.25 | 1.25 | 0.3 | >160.0 | 40.0 | 2.5 |
| F-31 | 0.6 | 0.6 | 0.15 | 0.3 | 1.25 | 0.3 | 0.3 | >160.0 | 20.0 | 2.5 |
| CH-1 | 2.5 | 1.25 | 2.5 | 0.6 | 0.15 | 0.3 | 0.3 | 80.0 | 40.0 | 0.6 |
| CH-2 | 5.0 | 2.5 | 2.5 | 0.3 | 0.3 | 0.3 | 0.3 | 80.0 | 20.0 | 0.3 |
| CH-5 | 2.5 | 2.5 | 1.25 | 0.3 | 0.6 | 0.6 | 0.6 | 160.0 | 40.0 | 0.6 |
| CH-6 | 1.25 | 1.25 | 0.3 | 0.3 | 2.5 | 1.25 | 0.6 | >160.0 | 10.0 | 2.5 |
| Average | 3.43 | 1.74 | 1.45 | 0.53 | 0.67 | 0.63 | 0.71 | | | 2.89 |

*Chloramphenicol, oleandomycin, and bacitracin.* These drugs about equally effective on both the pre-1940 and recent isolates. Hirsch and Finland (1960), however, reported a slight decrease in chloramphenicol susceptibility between 1949 and 1959.

*Oleandomycin-tetracycline.* This "combination" drug was only slightly more effective on the recent isolates than on the pre-1940 strains. This corresponds with the results obtained with tetracycline alone.

*Polymyxin B.* Susceptibility to this drug was low in both groups of gonococci. Of the 15 pre-1940 strains, 5 required 80 units/ml or more for complete inhibition, while 13 of the 15 recent isolates required this concentration.

*Sulfisoxazole.* In general, the recently isolated strains were slightly less susceptible to sulfisoxazole than the pre-1940 strains. The majority of the pre-1940 strains required 5 to 10 μg/ml for complete inhibition, but most of the recent isolates required 20 to 40 μg/ml. Hirsch and Finland (1960) found that nearly all strains isolated in 1958 and 1959 were highly or moderately susceptible to sulfadiazine.

*Dihydrostreptomycin.* A striking difference in the susceptibility of the two groups of gonococci was seen with this drug. The pre-1940 strains exhibited an average MIC of 0.28 μg/ml as compared to an average MIC of 2.9 μg/ml for the recent isolates, a 10-fold difference. Similar observations with streptomycin have been reported by other workers (Hirsch and Finland, 1960; Reyn and Bentzon, 1961).

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## Literature Cited

Cradock-Watson, J. E., R. A. Shooter, and C. S. Nicol. 1958. Sensitivity of strains of gonococci to penicillin, sulphathiazole, and streptomycin. Brit. Med. J. 1:1091-1092.

Curtis, F. R., and A. E. Wilkinson. 1958. A comparison of the in vitro sensitivity of gonococci to penicillin with the results of treatment. Brit. J. Venereal Diseases **34**:70-82.

Fairbrother, R. W. 1961. Resistance of gonococci to penicillin. Lancet **2**:226-230.

Gerhardt, P., and C. G. Heden. 1960. Concentrated culture of gonococci in clear liquid medium. Proc. Soc. Exptl. Biol. Med. **105**:49-51.

Hirsch, H. A., and M. Finland. 1960. Susceptibility of gonococci to antibiotics and sulfadiazine Am. J. Med. Sci. **239**:41-50.

Odegaard, K. 1960. Sensitivity in vitro of gonococci to three penicillins. Acta Dermato-Venerol. **40**:504-507.

Reyn, A., and M. W. Bentzon. 1961. Possible effects of antibiotic treatment on the sensitivity and growth requirements of *Neisseria gonorrhoeae*. Bull. World Health Organization **24**:333-342.

Reyn, A., B. Korner, and M. W. Bentzon. 1958. Effects of penicillin, streptomycin and tetracycline on *N. gonorrhoeae* isolated in 1944 and in 1957. Brit. J. Venereal Diseases **34**:227-239.

Thayer, J. D., F. W. Field, M. I. Perry, J. E. Martin, and W. Garson. 1961. Surveillance studies of *Neisseria gonorrhoeae* sensitivity to penicillin and nine other antibiotics. Bull. World Health Organization **24**:327-331.

Thayer, J. D., W. Garson, and W. Deacon. 1960. Gonococcus - procedures for isolation and identification, p. 23-27. U.S. Public Health Service. Publication no. 499. U.S. Government Printing Office, Washington, D.C.

# EFFECT OF TETRACYCLINE ON BONE GROWTH IN THE PREMATURE INFANT

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Subsequent to the observations of Regna et al. (1951) and Albert (1953) that tetracyclines form complexes with several heavy metals, Andre (1956) demonstrated that tetracycline accumulates in the mineralized portion of growing bones and teeth of mice. It was also shown (Fillippi and Mela, 1957) that daily administration of 5 mg of tetracycline to pregnant mice resulted in the production of several skeletal deformities in the offspring of these animals. The studies of Rall et al. (1957) demonstrated the localization of a fluorophore of tetracycline in tumors as well as in bone. Further experimental and clinical evidence (Milch, Rall, and Tobie, 1957, 1958) indicated that the tetracyclines accumulate and are retained for only 24 to 48 hr in soft tissues, but persist for weeks to months in the regions of new bone formation. It has also been demonstrated by these and other investigators that the accumulation of tetracycline fluorescence in mineralizing tissues is unrelated to sex or route of administration. There is, however, a relationship between bone fluorescence and bone age since, deposition of tetracycline has been found to be most intense in young growing bone, localizing particularly in the metaphyseal region just below the provisional zone of calcification, in uncalcified ossification centers, and at the periosteal and endosteal surfaces of the diaphysis. Localization in adult bone is relatively poor, producing a cream-colored fluorescence of slight intensity. Tetracycline fluorescence has also been observed in bone callus following trauma, in intrinsic or metastatic neoplasms (Milch et al., 1957), in the teeth of weanling rats (Bevelander, Rolle, and Cohlan, (1961), and in experimental ulcers and regenerating tissue in animals (Hakkinen and Hataiala, 1959).

The effect of tetracycline on the skeleton of the Sand Dollar larva (Bevelander, Goldberg, and Nakahara, 1960*a*) and the chick embryo (Bevelander, Nakahara, and Rolle, 1960*b*) has also been

reported. Rearing the invertebrate Sand Dollar larva in sea water containing tetracycline produced specimens reduced in size and with disturbance of body form and reduction of bony development to vestigial skeletal rods. The introduction of 2.5 mg of tetracycline into the yolk sac of an 8-day-old chicken embryo resulted in deposition of the tetracycline fluorophore in the mineralized component of the developing skeleton, with a subsequent marked reduction in expected embryo size. The bones of the tetracycline-treated chick embryos were bowed and reduced in size; they also revealed inhibition of mineralization and abnormal calcification of the cartilaginous matrix.

Since the tetracyclines are known to traverse the human placental barrier (Charles, 1954), it seemed of more than academic interest to ascertain the effect of tetracycline administration on the human fetal and infant skeleton.

## Materials and Methods

Fortuitously, in March 1961 a 900-g premature infant was born to a 24-year-old primipara, who had been given 1 g of oral tetracycline daily for 3 weeks prior to delivery (for premature rupture of the membranes). The infant succumbed within 24 hr after delivery. At autopsy, death was ascribed to prematurity. Specimens of several bones (femur, rib, and mandible) were obtained for processing and examination according to a technique previously described (Bevelander et al., 1960*b*). Since it is impractical to acquire statistically significant data on fetal bone growth in utero, an indirect approach was utilized. This was accomplished by measuring the effect produced by the oral administration of tetracycline on the linear growth of the fibula of the premature infant.

Linear growth of the fibula was determined by a modification (Day and Silverman, 1952) of the Maresch and Deming (1939) X-ray technique, in which growth of the fibula is measured by placing sharp calipers at the diagonally opposite ends of the fibula shadow. The calipers are then applied to the scale of a millimeter rule. To reduce technical error, all films were taken by the same technician at a constant target-film distance and exposure. For each determination, two films of both the left and right fibula were made. The infant was removed from the table and replaced for each exposure. Each time a measurement was made the calipers were opened wide and then brought to the corners of the fibula. To further eliminate bias, measurements were made in the blind at the termination of the experiment. Each linear determination represents the average of four measurements.

Six apparently normal premature infants exposed to the same routine care and feeding were studied over a 30-day period. Birth weights varied from 1,140 to 1,630 g. Age at the first X ray varied

from 5 to 36 days. The lower extremity of each infant was X-rayed at the beginning of a 9 to 12 day control period. At the end of this period, another set of films was taken to establish a normal growth rate for each fibula. Immediately after the second set of films, tetracycline (Achromycin Pediatrics Drops, Lederle) at a level of 100 mg/kg per day, divided into four doses, was administered orally to each infant immediately before a feeding. After 9 to 12 days of treatment, a third set of films was taken and drug therapy was discontinued. Final fibula films were taken 9 to 10 days after cessation of tetracycline administration. Each fibula was measured as described and growth rate per day calculated.

## Results

*Fetal skeleton.* We examined sections of the human fetal rib, femur, mandible, and a deciduous tooth. In each of the skeletal structures, we observed an intense yellow-gold fluorescence in a considerable portion of the mineralized structure (Fig. 1 and 3). Although we could not accurately ascertain whether the process of mineralization in these bones was inhibited by the drug, the second part of this study would indicate that this is probably true. In any event, the localization of the fluorophore appears to be confined to the areas of bone most recently mineralized. This is well demonstrated by comparing the areas of bone fluorescence in Fig. 1, a section of femur photographed in ultraviolet light, with an adjacent section of the femur (Fig. 2) treated according to the Von Kossa method, a specific technique for demonstrating the sites of mineral deposition in developing bone (Bloom and Bloom, 1940). The regions of bone showing fluorescence in the ultraviolet photograph (Fig. 1) correspond with the areas of recent mineral deposition, as seen in the Von Kossa preparation (Fig. 2). It is evident from these observations that large amounts of tetracycline localize in the human fetal skeleton after maternal oral administration in the third trimester.

*Linear fibula growth.* Table 1 summarizes the fibula growth rates observed in the control, tetracycline, and recovery periods in the six premature infants studied. Fibula growth rate during the control period ranged from 0.17 to 0.20 and from 0.17 to 0.25 mm/day in the right and left fibula, respectively. During the tetracycline period, growth rate was depressed to values of 0.08 to 0.12 and 0.09 to 0.17 mm/day, with an average of 0.11 and 0.12 mm/day in the right and left fibula, respectively. After cessation of tetracycline administration, the fibulas returned to a growth rate which was higher in most instances than their respective control rates.

The fibula growth rates of both limbs of each infant are plotted in Fig. 4. Mean values derived from this chart are 0.19, 0.11, and

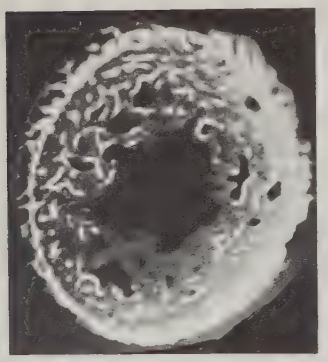

FIG. 1. *Ultraviolet photograph of cross-section of femur of a 900-g premature infant with transplacentally acquired tetracycline deposition.*

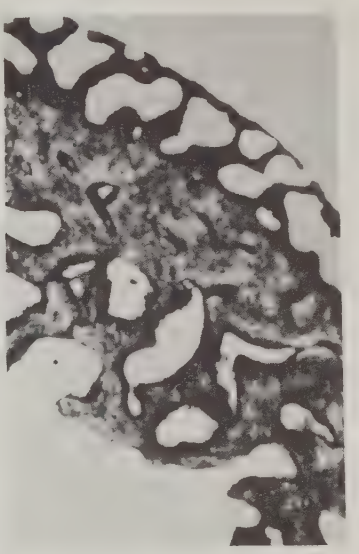

FIG. 2. *Adjacent section of same femur treated with the Von Kossa method for mineral localization. Note that regions showing presence of fluorophore in Fig. 1 (the white areas) correspond with those areas which are black (mineral deposition) in the Von Kossa preparation shown here.*

FIG. 3. *Section of rib photographed with ultraviolet light, showing tetracycline fluorescence (white areas).*

TABLE 1. *Summary of data of fibula growth rate in six premature infants.*

| Infant | Birth weight | First X ray | | Control | | Tetracycline | | Recovery | |
|---|---|---|---|---|---|---|---|---|---|
| | | Age | Weight | Right | Left | Right | Left | Right | Left |
| | *g* | *days* | *g* | *mm/day* | | *mm/day* | | *mm/day* | |
| Ma. | 1630 | 26 | 1720 | 0.17 | 0.20 | 0.12 | 0.10 | 0.23 | 0.22 |
| Ri. | 1140 | 32 | 1550 | 0.19 | 0.25 | 0.12 | 0.10 | 0.24 | 0.23 |
| Mat. | 1170 | 36 | 1820 | 0.17 | 0.19 | 0.10 | 0.09 | 0.23 | 0.22 |
| Wi. | 1575 | 11 | 1570 | 0.20 | 0.23 | 0.12 | 0.15 | 0.24 | 0.20 |
| Go. | 1450 | 5 | 1365 | 0.18 | 0.17 | 0.12 | 0.17 | 0.21 | 0.20 |
| Ra. | 1580 | 15 | 1550 | 0.20 | 0.19 | 0.08 | 0.12 | 0.23 | 0.23 |

0.22 mm/day for the control, tetracycline, and recovery periods, respectively. Figure 5 illustrates the per cent deviation of each fibula growth rate in the tetracycline and recovery periods, as compared to its respective control rate. Except for one instance, the fibulas showed a 30 to 60% depression of growth rate. During the recovery period, however, the average rate of fibula growth was 13% above the average control rate.

Individual fibula growth rate did not appear to be influenced by birth weight, chronological age, weight when first X-rayed, or weight gain per day during the study period. Two of the six infants developed mild, self-limited oral *Candida albicans* infections.

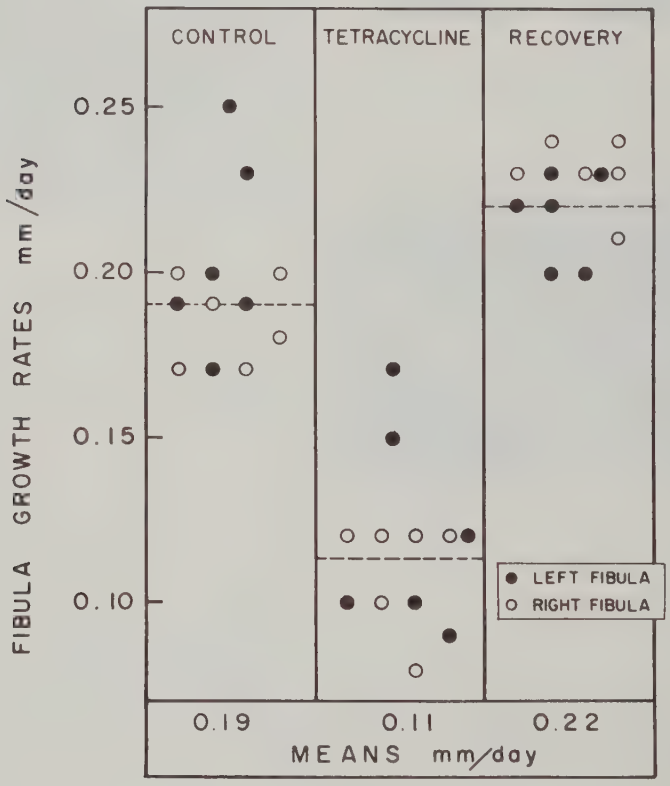


FIG. 4. *Fibula growth rates of six premature infants during control, tetracycline, and recovery periods.*

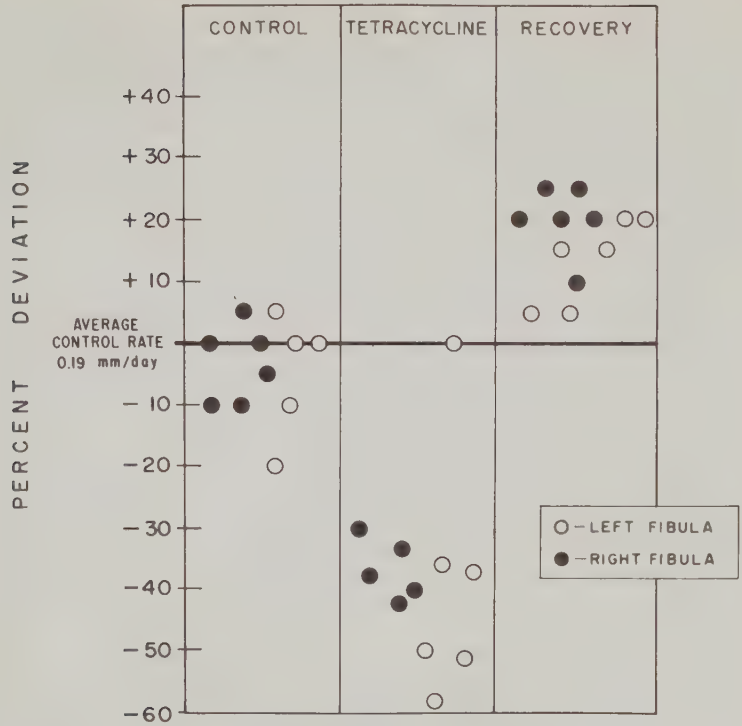


FIG. 5. *The control panel plots the per cent deviation of each fibula from the average control rate (mm/day). Each circle in the tetracycline and recovery panels represents the per cent deviation of each fibula growth rate (mm/day), as compared to its own control rate.*

## Discussion

The roentgen technique for evaluation of fibula growth in the premature infant has been reported in a control study by Day and Silverman (1952), and in an investigation of the effect of ACTH (Silverman, Day, and Blodi, 1951) on fibula growth in the premature. Snyderman and Holt (1961) utilized the same technique in evaluating the effect on skeletal growth of high caloric feeding. In eight normal premature infants, Day and Silverman observed an average fibula growth rate of 0.20 mm/day. Snyderman and Holt (1961) also recorded an average growth rate of 0.20 mm/day in the fibulas of 11 control premature infants, and rates of 0.20 and 0.18 mm/day in the left and right fibula, respectively, of 11 premature infants on high caloric feedings.

Our control values of 0.20 and 0.18 mm/day are almost identical with those cited, and are a reflection of the reproducibility of this technique for evaluating fibula growth. In our measurements and in those of Day and Silverman (1952), the maximal difference between duplicate pairs of measurements was 0.6 mm. Since the length of all bones measured ranged between 50 and 60 mm, the maximal difference between duplicate measurements represents approximately 1% of the average length of the bones measured, and the coefficient of variation was calculated to be 0.22%.

There was no evidence of growth disturbance or malformation in the human fetus studied. However, the potential effect on the fetal skeleton of transplacentally acquired tetracycline obviously requires further clinical observation. As for the premature infant, the transient nature of tetracycline-induced inhibition of fibula growth would suggest that the short-term use of tetracycline is probably without hazard. However, the effects of long-term tetracycline therapy on the infant skeleton awaits further investigation.

It may be stated, from our observations of premature infants, laboratory animals, and one fetal skeleton, that (i) transplacentally acquired tetracycline deposits in the fetal skeleton; (ii) tetracycline administered to the premature infant inhibits linear bone growth; and (iii) this inhibition of bone growth is apparently reversible. Whether this reversibility is dependent on dose or duration of therapy merits further investigation.

The above observations add to the increasing evidence that drugs and biologicals may have unusual effects when administered either directly to the premature and full-term neonate or indirectly to the fetus, via the placenta. Unfortunately, testing of pharmaceuticals prior to wide distribution rarely includes toxicity studies in the fetus or newborn.

## Acknowledgment

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## Literature Cited

Albert, A. 1953. Avidity of tetracycline and Aureomycin for metallic cations. Nature **172**:201.

Andre, T. 1956. Studies on the distribution of tritium–labeled dihydrostreptomycin and tetracycline in the body. Acta Radiol., Suppl. **142**:1-89.

Bevelander, G., I. Goldberg, and H. Nakahara. 1960*a*. The effect of tetracycline on skeletal development in the larval Sand Dollar *(Echinarachnius Parma)*. Arch. Oral Biol. **2**:127-130.

Bevelander, G., H. Nakahara, and F. K. Rolle. 1960*b*. The effect of tetracycline on the development of the skeletal system of the chick embryo. Developmental Biol. **2**:298-312.

Bevelander, G., G. K. Rolle, and S. Q. Cohlan. 1961. The effect of the administration of tetracycline on the development of teeth. J. Dental Research **40**:1020-1024.

Bloom, W., and M. A. Bloom. 1940. Calcification and ossification. Calcification of developing bones in embyronic and new born rats. Anat. Record **78**:497-524.

Charles, D. J. 1954. Placental transmission of antibiotics. J. Obstet. Gynaecol. Brit. Empire **61**:750-757.

Day, R., and W. A. Silverman. 1952. Growth of the fibula of premature infants as estimated in roentgen films: a method for assessing factors promoting or inhibiting growth. Études Neonatales **1**:1-4.

Fillippi, B., and V. Mela. 1957. Malformazioni congenite facciali e degli arti da tetraciclina. Minerva chir. **12**:1106-1110.

Hakkinen, I., and K. Hataiala. 1959. Fluorescence of tetracycline in experimental ulcers and regenerating tissue injuries. Ann. Med. Exptl. et Biol. Fenniae (Helsinki) **37**:115-120.

Maresch, M. M., and J. Deming. 1939. The growth of the long bones in 80 infants: roentgenograms versus anthropometry. Child. Develop. **10**:91-106.

Milch, R. A., D. P. Rall, and J. E. Tobie. 1957. Bone localization of the tetracyclines. J. Natl. Cancer Inst. **19**:87-92.

Milch, R. A., D. P. Rall, and J. E. Tobie. 1958. Fluorescence of tetracycline antibiotics in bone. J. Bone and Joint Surg. **40a**:897-910.

Rall, D. P., T. L. Loo, M. Lane, and M. G. Kelly. 1957. Appearance and persistence of fluorescent material in tumor tissue after tetracycline administration. J. Natl. Cancer Inst. **19**: 79-85.

Regna, P. P., I. A. Solomons, K. Murai, A. E. Timreck, K. J. Brunings, and W. A. Iazier. 1951. The isolation and general properties of Terramycin and Terramycin salts. J. Am. Chem. Soc. **73**:4211-4215.

Silverman, W. A., R. L. Day, and F. C. Blodi. 1951. Inhibition of growth and other effects of ACTH in premature infants. Pediatrics **8**:177-191.

Snyderman, S. E., and L. E. Holt, Jr. 1961. The effect of high caloric feeding on the growth of premature infants. J. Pediat. **58**:237-240.

# EXCRETION OF METHICILLIN IN HUMAN BILE

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Methicillin is a synthetic antibiotic which is made from 6-aminopenicillanic acid (Rolinson et al., 1960). This compound is effective against all strains of *Staphylococcus* and is not affected by penicillinase (Gourevitch et al., 1961; Roberts, Allen, and Kirby, 1961). The drug must be administered parenterally; therefore, if it is to be effective against staphylococcal infections in the bile and in the gastrointestinal tract, it must be excreted by the liver cells into the bile. Stewart, Harrison, and Holt (1960) showed that bile obtained by means of a duodenal tube in one patient contained 3 to 4.5 $\mu$g/ml of antibiotic in bile samples that were taken at 75, 80, and 90 min after a single intramuscular injection of 1 g of the drug. Our study was undertaken to determine the level of methicillin in the bile at different time intervals and to see if the antibiotic is concentrated in the gall bladder bile.

## Materials and Methods

Bile was collected from 11 patients with normal liver function and from one patient with impaired liver function. The bile was collected in eight patients at the time of surgery in the upper abdomen. It was collected from the gall bladder and the hepatic ducts by means of needle aspiration. In two patients, no gall bladder bile was obtainable. The time interval for bile collection, following a single intramuscular dose of 1 g of methicillin, was as shown in Table 1. Serum levels were obtained at the beginning and at the end of the operative procedure, and three patients had bile collected at 2-hr intervals from a T tube in the common bile duct.

All antibiotic levels were determined by the doubling tube dilution technique using microorganisms isolated from the clinical laboratory (Preston et al., 1960).

TABLE 1. *Antibiotic levels in bile after a 1-g, intramuscular dose of methicillin*

| Patient | Time* | Serum | Methicillin level | |
|---|---|---|---|---|
| | | | Bile from gall bladder | Bile from common duct |
| | *hr* | *μg/ml* | *μg/ml* | *μg/ml* |
| S. C. H. | 1.5 | 12.5 | | |
| | 2.5 | | 0.4 | 0.8 |
| | 4.0 | 3.2 | | |
| R. O. B. | 0.75 | 3.0 | | |
| | 2.0 | | 1.6 | <0.4 |
| | 4.0 | 0.8 | | |
| R. I. O. | 1.0 | 3.2 | | |
| | 2.0 | | <0.8 | <0.8 |
| | 3.5 | 3.2 | | |
| Mc. G. | 2.0 | 5.0 | | |
| | 2.75 | | 5.0 | 5.0 |
| | 4.5 | 0.5 | | |
| H. O. U. | 2.0 | 5.0 | | |
| | 2.5 | | 20.0 | 10.0 |
| | 4.5 | 0.5 | | |
| T. I. L. | 8.0 | <0.15 | | |
| | 8.5 | | 20.0 | |
| H. A. R. | 8.0 | <0.16 | | |
| | 9.0 | 1.0 | | <0.08 |
| P. E. T. | 1.0 | 10.0 | | |
| | 2.0 | 5.0 | | |
| | 3.0 | 5.0 | | |
| | 4.0 | 2.5 | | |
| | 5.0 | 2.5 | | |
| | 6.0 | 1.25 | | |
| | 6.75 | | 38.0 | 5.0 |

*Time is recorded as the nearest fraction of the hour after the drug dose.

## Results

In three patients from whom bile was obtained from the gall bladder and the common hepatic duct at approximately 2 hr, there was less than a therapeutic level in either bile sample. We consider a therapeutic level as 4 to 5 times the sensitivity level of the microorganisms. One patient in whom bile was obtained at 2.5 hr had 20 μg/ml of antibiotic in the gall bladder bile and 10 μg/ml in the hepatic bile. Another patient had 5.0 μg/ml of antibiotic in the gall bladder and hepatic bile at 2.75 hr (Table 1).

One patient was given a single dose of 1 g of methicillin 8.5 hr prior to obtaining bile from the gall bladder. He had less than 0.15 μg/ml antibiotic in the serum and 20 μg/ml of antibiotic in the gall bladder bile at that time. No bile was obtained from the common bile duct. Another patient (H. A. R.) had only common duct bile studied 9 hr after a single intramuscular dose of the drug. It contained less than 0.08 μg/ml of antibiotic activity. A patient studied

6.75 hr after a single dose of methicillin had 38 and 5 μg/ml of antibiotic activity in the gallbladder and common duct bile, respectively.

Three patients with T tubes in the common duct were studied at intervals following a single intramuscular 1-g dose of methicillin. The bile and serum levels are shown in Table 2.

TABLE 2. *Antibiotic levels in common duct bile after a 1-g intramuscular dose of methicillin*

| Patient | Time | Concentration | | Volume of bile |
|---|---|---|---|---|
| | | Serum | Bile | |
| | *hr* | *μg/ml* | | *ml* |
| M. A. L. | 1 | 6.4 | | |
| | 2 | 6.4 | <0.8 | 20 |
| | 3 | 6.4 | | |
| | 4 | 3.2 | <0.8 | 20 |
| | 5 | 1.6 | | |
| | 6 | 0.4 | <0.8 | 25 |
| | 8 | | <0.8 | 20 |
| | 10 | | 1.6 | 15 |
| | 12 | 0.4 | <0.8 | 25 |
| H. E. I. | 1 | 12.8 | | |
| | 2 | 6.4 | <1.6 | 6 |
| | 3 | 1.6 | | |
| | 4 | <0.8 | 1.6 | 15 |
| | 5 | <0.8 | | |
| | 6 | <0.8 | 3.2 | 2 |
| | 8 | | 3.2 | 20 |
| | 10 | | 3.2 | 12 |
| | 12 | <0.8 | 3.2 | 10 |
| P. A. S. | 1 | 10.0 | | |
| | 2 | 2.5 | <0.3 | 75 |
| | 4 | 1.2 | 0.3 | 75 |
| | 6 | <0.6 | 1.2 | 60 |
| | 8 | <0.6 | 2.5 | 60 |
| | 10 | | 2.5 | 60 |
| | 12 | | 2.5 | 75 |

Patient M. A. L. had a carcinoma of the head of the pancreas with hepatic metastases. The alkaline phosphatase was 56.2 King-Armstrong units and the bilirubin was elevated in spite of T-tube drainage. He did not excrete methicillin in the bile obtained from the T tube (Table 2).

A patient (H. E. I.) with normal liver function, but a small output of bile through the T tube, had a peak level of only 3.2 μg/ml of methicillin in the bile at 6 hr (Table 2). The test organism was sensitive to 0.8 μg/ml in the serum and 1.6 μg/ml in bile; therefore, the bile levels were not considered to be within a therapeutic range.

The third patient with a T tube (P. A. S.) had 1.2 μg/ml in the common duct bile at 6 hr; at 8 hr he had 2.5 μg/ml, and this level lasted through the 12-hr collection period.

## Discussion

When a single 1-g dose of methicillin is given intramuscularly to a patient with normal liver function, it appears in the bile, at the therapeutically effective levels, in 2 to 6 hr. In one patient with impaired liver function, no significant level of methicillin was reached in the bile during the 12 hr of the study.

Since parenterally administered antibiotics can only reach the intestinal fluid through the bile, this drug would be effective against sensitive microorganisms in the intestinal tract and in the bile. However, with impaired liver function, the antibiotic might not be excreted in the bile.

## Acknowledgment

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## Literature Cited

Gourevitch, A., G. A. Hunt, T. A. Pursiano, A. J. Moses, and J. Lein. 1961. Microbiological studies on dimethoxyphenyl penicillin, p. 4-16. *In* P. A. Bunn, [ed.], Symposium on the new dimethoxyphenyl penicillin. State University of New York, College of Medicine, Syracuse.

Preston, F. W., M. Silverman, G. C. Henegar, and E. Neveril. 1960. The excretion of kanamycin in bile and pancreatic fluid. Antibiotics Ann. 1959-1960, p. 857-861.

Roberts, C. E., Jr., J. D. Allen, and W. M. M. Kirby. 1951. Laboratory and clinical studies with penicillin X-1497. Clin. Pharmacol. Therap. **2**:70-79.

Rolinson, G. N., S. Stevens, F. R. Batchelor, J. C. Wood, and E. B. Chain. 1960. Bacteriological studies on a new penicillin - BRL 1241. Lancet **2**:564-567.

Stewart, G. T., P. M. Harrison, and R. J. Holt. 1960. Microbiological studies on sodium 6-(2,6 dimethoxybenzamido) penicillinate monopydrate (BRL 1241) in vitro and in patients. Brit. Med. J. **2**:694-699.

# CYCLOSERINE IN THE TREATMENT OF HUMAN NOCARDIOSIS

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Three patients with nocardiosis have been treated with cycloserine. In two it was the primary therapy and in the other it was used after the patient manifested allergy to sulfonamides. In two cases the use of cycloserine coincided with clinical improvement, and during the period of follow-up there was no evidence of active disease. Another patient died 12 days after beginning treatment, apparently as a result of an acute myocardial infarction. Autopsy failed to disclose the presence of *Nocardia asteroides* in the lungs, bone marrow, or spleen. This limited experience suggests that cycloserine might have a place in the treatment of nocardiosis and that further studies are indicated.

## Case Reports

*Case no. 1.* J. B. is a 68-year-old man admitted to the Dermatology Service in June 1958 because of a generalized bullous eruption of 3 weeks duration. The diagnosis of pemphigus vulgaris was confirmed by skin biopsy. The chest X-ray film was normal. The patient was placed on prednisone, and after a satisfactory remission was followed in the outpatient department.

In September 1958 he complained of a productive cough with brownish, blood-tinged sputum present for 4 days, but denied other symptoms. Chest X-ray films revealed a hazy density in the right apex and the patient was again hospitalized. Except for the skin disease and a known incoordination of speech and gait present for many years, there were no unusual physical findings. Skin tests with histoplasmin, coccidioidin, and tuberculin were negative. The initial sputum smear revealed acid-fast bacilli, and the patient was placed on isaniazid and sodium *p*-amino salicylate (antituberculosis therapy). Sputum cultures revealed *N. asteroides* on two occasions. Several

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sputum cultures were negative for tubercle bacilli. By 10 October 1958 he had lost 25 lb, and his cough continued. He was placed on cycloserine (250 mg daily), and the antituberculosis medications were stopped. The cycloserine was gradually increased to 750 mg daily, and hydrocortisone (30 mg daily) was continued. Two weeks after beginning cycloserine there was marked decrease in the cough and in the amount of sputum, and he began to gain weight. By the time he was discharged from the hospital, 1 month after beginning cycloserine therapy, he had gained 16 lb. He continued cycloserine (750 mg daily) for a total of 3 months until 9 January 1959, at which time he was asymptomatic and had regained his usual weight of 150 lb. All sputum cultures after beginning cycloserine were negative. Two years after therapy was stopped, a chest X ray (Fig. 1) revealed haziness in the right apex, which has not changed since the onset of his pulmonary disease. There was no evidence of toxicity to cycloserine, even in the presence of the underlying neurological disorder.

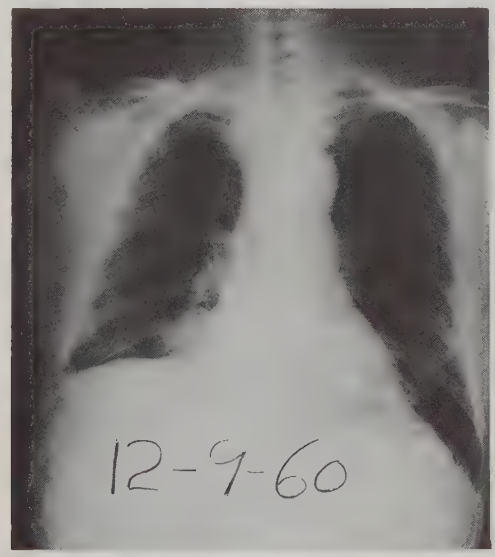


FIG. 1. *Chest X-ray of patient no. 1 (J. B.) approximately 2 years after stopping cycloserine.*

*Case no. 2.* W. H. was a 70-year-old man who entered University Hospital 28 March 1960 because of increasing shortness of breath and chronic productive cough. The patient was in acute respiratory distress, with distended neck veins and edema of the lower

extremities. Coarse inspiratory and expiratory rales were heard throughout the lungs. The heart was enlarged and an apical systolic murmur was heard. The liver edge was discovered 3 cm below the right costal margin, but the spleen was not palpable. Hemoglobin concentration was 12.3 g per 100 ml, and the white blood cell count was 13,350 per $mm^3$ with 80% neutrophils. Skin tests with tuberculin, histoplasmin, and coccidioidin were negative. Sputum smears and cultures for tubercle bacilli were negative. A chest X-ray film revealed extensive infiltrative changes bilaterally, with cavitation in the right midlung field (Fig. 2). The patient responded slightly to treatment for congestive failure. On 6 May 1960, after *N. asteroides* was reported from the sputum and bone-marrow cultures, he was placed on cycloserine (250 mg daily). Six days after beginning therapy, cycloserine was increased to 500 mg daily, and the patient went to his home which was within a few blocks of the hospital.

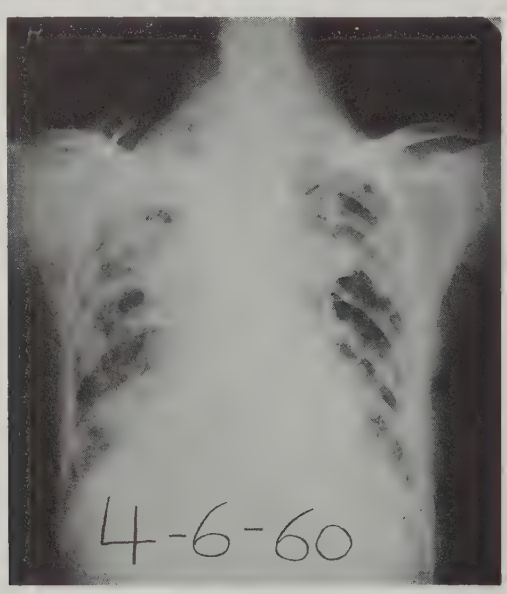


FIG. 2. *Chest X-ray of patient no. 2 (W. H.) upon admission to the hospital. Note the cavity in the right midlung field.*

Six days after discharge and 12 days after cycloserine was begun, he experienced sudden, severe chest pain and extreme dyspnea and died en route to the hospital. Autopsy revealed thickened pleural surfaces, palpable nodules in the apices of the lungs, and multiple

small cavities throughout the upper lobes of both lungs. There was evidence of perisplenitis. The liver appeared normal except for passive congestion. Microscopic examination of the lungs revealed inflammatory cells and a few giant cells in the walls of cavities. The parenchyma of the lungs elsewhere suggested "foreign-body type reaction." Brown-Brenn stains revealed no organisms, and stains for acid-fast bacilli were negative. The hilar lymph nodes were essentially unaltered. The posterior myocardium of the left ventricle exhibited foci of leukocytes, with occasional basophilic degeneration of muscle fibers compatible with a diagnosis of acute myocardial infarction. Cultures of lung cavities, bone marrow, and spleen were negative for *N. asteroides*. The brain was not examined.

*Case no. 3.* L. W. is a 51-year-old lumber yard worker who had been in good health all his life. In April 1960 he was hospitalized because of malaise, fatigueability, and cough productive of one-fourth cup of yellowish sputum daily. These symptoms had been present for 6 weeks, and were associated with a 17-lb weight loss. He was dyspneic at rest, but the physical examination was not remarkable except for dry rales in the left side of the chest. Skin tests with tuberculin, histoplasmin, and coccidioidin were negative. Blood and urine studies were normal. Cultures of sputum for tubercle bacilli were repeatedly negative. A smear of the sputum on one

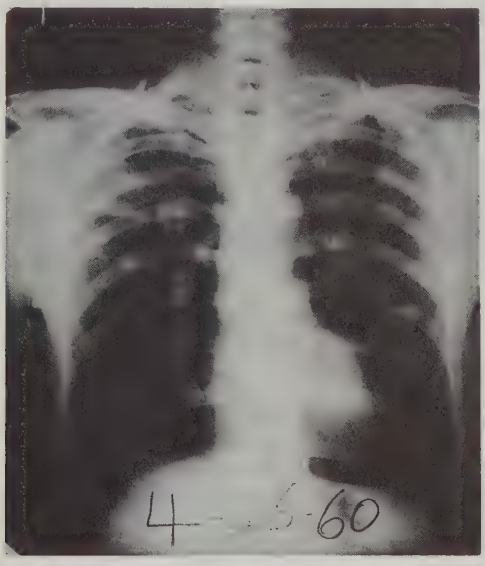

FIG. 3. *Chest X-ray of patient no. 3 (L. W.) upon admission to the hospital.*

occasion revealed a few acid-fast organisms, and cultures subsequently revealed *N. asteroides*. Chest X-ray film on admission to the hospital showed extensive bilateral infiltrates (Fig. 3). He was placed on sulfadiazine (4 g daily), but after 10 days developed a generalized skin rash and the medication was stopped. He remained off therapy for 2 weeks and complained of increased malaise. Sulfisoxazole therapy was attempted but had to be abandoned after 1 week because of swelling of wrists and hands and blistering of his skin. An X-ray film on 26 July 1960 revealed an apparent increase in the infiltrate in the right lower lung field (Fig. 4). In August 1960

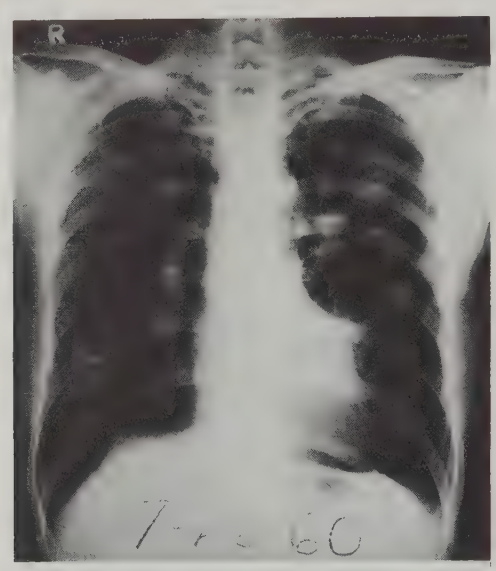


FIG. 4. *Chest X-ray of patient no. 3 (L. W.) after a short trial of sulfonamides and prior to cycloserine therapy.*

he was placed on cycloserine (500 mg daily). After 1 week there was gradual improvement, with decreased cough, decreased dyspnea, and increased strength. An X ray 1 month after beginning cycloserine (Fig. 5) revealed considerable clearing. Cycloserine was continued for 7 months, until 23 March 1961. Four months after beginning cycloserine his weight had returned to normal and there was continued improvement. He has returned to work in a lumber yard and, except for dyspnea upon unusual exertion, is free of symptoms.

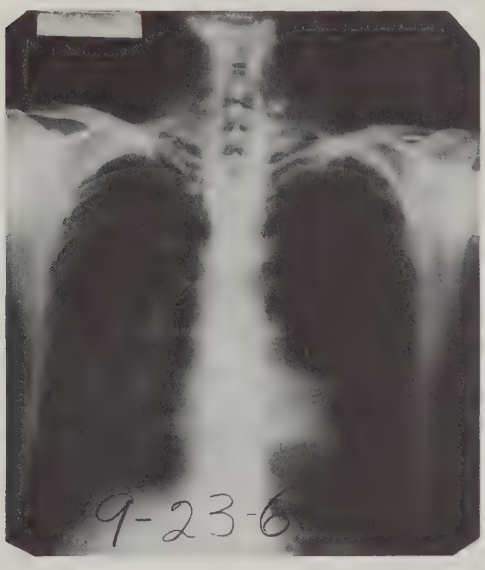


FIG. 5. *Chest X-ray of patient no. 3 (L. W.) 1 month after beginning cycloserine.*

## Discussion

These three patients appear to have had proved infection with *N. asteroides.* All had lung disease and in all the organism was recovered from the sputum. One had disseminated disease with organisms in the bone marrow. All failed to react to the usual skin tests, and bacteriological search for other pathogens was not successful. Cycloserine was the only therapy given for nocardiosis in two patients and in the other it was used after the patient appeared to be hypersensitive to sulfonamide preparations. Improvement in the first and third patients began within 2 weeks after starting cycloserine, and continued gradually. At the time of the last follow-up there was no evidence of active infection. The second patient expired soon after beginning therapy, apparently from an acute myocardial infarct. Although possible, it seems unlikely that his death was the result of his disease or his medication. Members of his family present at the time of his death noticed no mental or neurological changes. It seems unlikely that any drug would sterilize chronically diseased tissues in only 12 days, but there were no organisms found in the tissues examined microscopically and bacteriologically.

Cycloserine was given to the first patient in an average dose of 750 mg daily for 3 months. None of the patients had side effects which could be ascribed to cycloserine. The drug was given in divided doses and did not exceed 1 g per day.

Drug susceptibility studies of the organisms recovered from two patients suggested in vitro resistance of greater than 100 $\mu$g per ml. This seems to support the concept of the lack of correlation between in vitro sensitivity and clinical response in this disease (Strauss, Kligman, and Pillsbury, 1951; Runyon, 1951; Sanford, Hatten, and Fordtran, 1958).

The duration of therapy in this study was based upon clinical response. Treatment was continued for a few weeks after no evidence of active disease could be observed. At the time of the last follow-up, patients no. 1 and 3, although free of symptoms, had roentgenological evidence of inactive pulmonary residue.

At the present time, the therapy of choice for nocardiosis still appears to be one of the sulfonamides. However, because of the apparent benefit derived in these three cases, further investigation with cycloserine in this disease seems indicated. This drug may prove useful in patients allergic to sulfonamides, in those who do not respond to sulfonamide administration, or in conjunction with sulfonamide therapy.

## Literature Cited

Runyon, E. H. 1951. *Nocardia asteroides:* studies of its pathogenicity and drug sensitivities. J. Lab. Clin. Med. **37**:713-720.

Sanford, J. P., B. E. Hatten, and J. S. Fordtran. 1958. Effectiveness of novobiocin and cycloserine on *Nocardia asteroides*. Antibiotics Ann. 1957-58, p. 22-26.

Strauss, R. E., A. M. Kligman, and D. M. Pillsbury. 1951. The chemotherapy of actinomycosis and nocardiosis. Am. Rev. Tuberc. **63**:441-448.

# THERAPEUTIC TRIAL OF CURATIVE DRUGS FOR SYSTEMIC FUNGUS DISEASE

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Three different therapeutic agents, the aromatic diamidines, amphotericin B, and antibiotic X5097C, have been reported to be effective in the treatment of North American (NA) blastomycosis, as well as other systemic mycoses. A new dimension has thus been added to the conduct of therapeutic trials in this group of diseases, and data from therapeutic trials need to be re-evaluated to determine their appropriateness to the new situation. In this paper and in the accompanying paper (Sutliff and Mills, 1962), we will consider some of the available data on the therapy of NA blastomycosis with aromatic diamidines and amphotericin B. The authors have had no experience with X5079C.

This paper will deal with data collected from the literature. All of the data from the English literature that are readily available to the authors were tabulated. These 135 cases, described in 50 reports (see Literature Cited), were studied: 81 cases treated with stilbamidine or hydroxystilbamidine, 39 cases treated with amphotericin B, and 15 cases treated with both agents. The number of cases ranged from 1 to 16 in each report. All of the data were obtained without controls, with relatively short follow-up periods. Only cases in which *Blastomyces dermatitidis* was demonstrated and in which the therapeutic agent was administered intravenously were tabulated.

It was sometimes assumed in these reports that the reader could compare the fatality data with a standard; some basic figures were therefore obtained, from two contrasting studies, for use in this analysis. The data of Martin and Smith (1939) were based on 117 systemic, proven and presumptive, cases; the data of Kunkel et al. (1954) were based on 36 systemic, proven and presumptive, cases from one clinic. The cases assembled from the literature had a fatality rate of 92% among those followed for at least 2 years, and the cases assembled in the clinic showed a fatality rate of only 23% among cases followed for at least 2 years. Speculation as to the cause of this discrepancy led to the description of a tendency of authors to report diseases such as NA blastomycosis, which were once believed rare, only after definite proof; this often required

report of autopsy findings, and thus a proportionately large number of fatal cases would be among the published cases. The knowledge that such a discrepancy is possible needs to be recognized in judging the significance of the data available in the literature. The importance of adequate follow-up in arriving at the ultimate result in a chronic disease, such as NA blastomycosis, was also illustrated in the references above; the selection of cases with 2-year follow-up resulted in an increase of 20% in the fatality rates in both series.

While making this critical analysis, it is also necessary to mention that most reports described a prompt effect of therapy on visible lesions, without exact data from untreated cases for comparison. Such an observation, without specific data but supported by photographs of a few cases, can be convincing. The tendency of untreated NA blastomycosis skin lesions to change slowly is well known. Therefore, the complete disappearance of a series of skin lesions in less than 1 month, as reported by Sutliff, Kyle, and Hobson (1954), can generally be accepted as adequate evidence of definite therapeutic effect. This effect, however, can not be considered synonymous with cure or arrest of the systemic disease.

TABLE 1. *Outcome of 135 cases of North American blastomycosis treated with aromatic diamidines, amphotericin B, or diamidines followed by amphotericin B*

| Outcome | Treatment | | | | |
|---|---|---|---|---|---|
| | Diamidines | | Amphotericin B | | Both |
| | *no.* | % | *no.* | % | *no.* |
| Arrested | 54 | 67 | 27 | 69 | 12 |
| Improved | 12 | 15 | 9 | 23 | 1 |
| Unimproved | 10 | 12 | 1 | 3 | 0 |
| Died | 5* | 6 | 2 | 5 | 2* |
| Total | 81 | | 39 | | 15 |

*One case died of cause other than North American blastomycosis.

Table 1 shows the outcome in 135 cases of NA blastomycosis reported in the literature. Mortality rates of 5 or 6% in the cases treated with the aromatic diamidines or amphotericin B alone were considerably lower than those reported in previous studies of nonspecifically treated cases. It was not fully established whether this apparent reduction in fatality rates was solely due to the effect of therapy because of the dearth of comparable data from untreated cases.

The smaller group of 15 cases treated first with diamidines and subsequently with amphotericin B had a different significance. The favorable result, shown in Table 1, in which 12 of 15 cases were arrested, followed the use of amphotericin B. All these cases had been unimproved after diamidine therapy. If the results of both

courses of therapy were added to the totals for their respective series of figures, the resulting data would tend to show that amphotericin B had a more favorable effect than diamidine therapy. One should consider, however, whether diamidine therapy might not be equally effective in amphotericin B failures if the opportunity for such observation had been presented.

In summary, the data in the literature have been interpreted as showing that both the aromatic diamidines and amphotericin B had favorable therapeutic effects. It is apparent that the methods that were used would be inadequate to make further distinctions, such as the degree to which the course of the disease in treated cases differed from the natural course of the disease, or to determine the comparative merits or special advantages of three different effective therapeutic agents.

## Literature Cited


Abernethy, R. S. 1959. Clinical manifestations of pulmonary blastomycosis. Ann. Internal Med. **51**:707-727.

Abernethy, R. S., and G. T. Jansen. 1960. Therapy with amphotericin B in N. A. blastomycosis. Ann. Internal Med. **53**:1196-1203.

Baum, G. L., and J. Scwartz. 1960. Clinical experience with amphotericin B. Antibiotics Ann. 1959-60, p. 638-43.

Burr, A. H., and T. R. Huffines. 1954. Blastomycosis of the prostate with miliary dissemination treated by stilbamidine. J. Urol. **71**:464-8.

Beard, H. W., J. H. Richert, and R. R. Taylor. 1960. The treatment of deep mycotic infections with amphotericin B with particular emphasis on drug toxicity. Am. Rev. Respiratory Diseases **81**:43-51.

Block, T. C., and H. M. Wilson. 1953. Coexistent pulmonary tuberculosis and systemic blastomycosis. Am. Rev. Tuberc. **68**:615-21.

Blodi, F. C., and W. C. Huffman. 1958. Cicatricial ectropion caused by cutaneous blastomycosis. Arch. Ophthalmol. **59**:459-62.

Carmody, E. J., and W. Tappan. 1959. Blastomycotic meningitis: report of a case successfully treated with amphotericin B. Ann. Internal Med. **51**:780-90.

Colsky, J. 1954. Treatment of systemic blastomycosis with 2-hydroxy-stilbamidine. A.M.A. Arch. Internal Med. **93**:796-801.

Cummins, C. R., B. Bairston, and L. A. Baker. 1953. Stilbamidine in the treatment of disseminated blastomycosis. A.M.A. Arch. Internal Med. **92**:98-107.

Curtis, A. C., and E. R. Harrell, Jr. 1952. Use of two stilbene derivatives (diethylstilbesterol and stilbamidine) in the treatment of blastomycosis. Arch. Dermatol. and Syphilol. **66**:676-90.

Derbes, V. J., J. D. Krafchuk. 1958. Response of N. A. blastomycosis to amphotericin B. Bull. Tulane Univ. Med. Fac. **17**:157-63.

Farrell, G. E. 1961. Blastomycosis of the sacrococcygeal area. Am. Surgeon **154**:90-92.

Fink, J. C., D. E. Vanderploeg, and M. P. Moursund. 1953. Stilbamidine in the treatment of cutaneous blastomycosis, report of a case. J. Am. Med. Assoc. **151**:1395-7.

Forsey, R. R., and R. Jackson. 1953. Toxic psychosis following use of stilbamidine in blastomycosis. Arch. Dermatol. and Syphilol. **68**:89-90.

Gordon, E. A., and W. B. Stewart. 1960. Therapy of N. A. blastomycosis. Can. Med. Assoc. J. **82**: 471-493.

Grandbois, J. 1958. The modern treatment of N. A. blastomycosis. Can. Med. Assoc. J. **79**:828-832.

Greer, A. 1960. N. A. blastomycosis of a nasal sinus. Diseases of the Chest **38**:454.

Harrell, E. R., F. C. Bocobo, and A. C. Curtis. 1955. A study of N. A. blastomycosis and its treatment with stilbamidine and 2-hydroxystilbamidine. Ann. Internal Med. **43**:1076-91.

Harrell, E. R., and A. C. Curtis. 1957. The therapy of N. A. blastomycosis with amphotericin B. Arch. Dermatol. and Syphilol. **76**:561-568.

Harrell, E. R., and A. C. Curtis. 1959. N. A. blastomycosis. Am. J. Med. **27**:750-766.

Kunkel, W. M., Jr., L. A. Weed, J. R. McDonald, and O. T. Clagett. 1954. N. A. blastomycosis, Gilchrist's disease: a clinicopathologic study of 90 cases. Surg. Gynecol. Obstet. **99**:1-26.

Lester, C. F., F. G. Conrad, and R. J. Atwell. 1958. Primary laryngeal blastomycosis. Am. J. Med. **24**:305-309.

London, I. D. 1956. Hydroxystilbamidine therapy of N. A. blastomycosis. Southern Med. J. **49**: 1098-1103.

Loudon, R. G., and R. A. Lawson. 1961. Systemic blastomycosis; recurrent neurological relapse in a case treated with amphotericin B. Ann. Internal Med. **55**:139-147.

McCauley, W. L. 1956. Is cutaneous blastomycosis a systemic disease? Arch. Dermatol. and Syphilol. **73**:560-563.

McLean, W. C., and G. S. Fitzhugh. 1959. Blastomycosis as encountered in the practice of otolaryngology. Southern Med. J. **52**:667-74.

Martin, D. S., and D. T. Smith. 1939. Blastomycosis (American blastomycosis, Gilchrist's disease). A review of the literature. Am. Rev. Tuberc. **39**:275.

Matsumoto, K. K., D. S. Amatusio, T. L. Lomasney, W. W. Ayres, and T. D. Cuttle. 1955. N. A. blastomycosis treated with pulmonary resection and stilbamidine. Am. J. Med. Sci. **229**: 172-179.

Myhr, L. B. 1954. Systemic blastomycosis: report of a case treated with stilbamidine. Am. Practitioner **5**:64-66.

Newcomer, V. D., T. H. Sternberg, E. T. Wright, R. M. Reisner, E. G. McNail, and L. J. Sorenson. 1960. The treatment of systemic infection with amphotericin B. Ann. N. Y. Acad. Sci. **89**: 221-239.

Ostfeld, A. M. 1953. Effect of stilbamidine on cutaneous blastomycosis. Am. J. Med. **15**:746-48.

Pariser, H., E. D. Levy, and A. J. Rawson. 1953. Treatment of blastomycosis with stilbamidine; report of a case. J. Am. Med. Assoc. **152**:127-130.

Rolnick, D., and G. O. Baumrucker. 1958. Genitourinary blastomycosis cases; case report and review of literature. J. Urol. **79**:315-23.

Schoenbach, E. B., J. M. Miller, M. Ginsberg, and P. H. Long. 1951. Systemic blastomycosis treated with stilbamidine: a preliminary report. J. Am. Med. Assoc. **146**:1317-1318.

Schoenbach, E. B., J. M. Miller, and P. H. Long. 1952. The treatment of systemic blastomycosis with stilbamidine. Ann. Internal Med. **37**:31-47.

Seabury, J. H., and H. E. Dascomb. 1958. Experience with amphotericin B for the treatment of systemic mycoses. A.M.A. Arch. Internal Med. **102**:960-976.

Seabury, J. H., and H. E. Dascomb. 1960. Experience with amphotericin B. Ann. N. Y. Acad. Sci. **89**:202-220.

Sivak, G. C., and R. Lich. 1956. Blastomycosis treated with stilbamidine with relapse. J. Urol. **76**:678-683.

Slaughter, J. C., Jr. 1954. Stilbamidine in the treatment of widely disseminated blastomycosis (with 2-year follow-up). Arch. Dermatol. and Syphilol. **70**:663-665.

Smith, D. T. 1953. Stilbamidine therapy for blastomycosis. G. P. **8**:(July) 69.

Snapper, F., and L. V. McVey. 1953. The treatment of N. A. blastomycosis with 2-hydroxystilbamidine. Am. J. Med. **15**:603-23.

Staab, W. J., Jr., and H. S. Van Ordstrand. 1958. Pulmonary blastomycosis: a report of a case treated with hydroxystilbamidine. Cleveland Clinic Quart. **25**:164-168.

Stillians, A. W., and H. E. Klemptner. 1953. Blastomycosis in a tuberculosis patient. J. Am. Med. Assoc. **153**:560-561.

Sutliff, W. D., and D. Mills. 1962. Chemotherapy and antibiotic therapy of North American blastomycosis cases observed from 2 to 10 years. Antimicrobial Agents and Chemotherapy - 1961, p. 000-000.

Sutliff, W. D., J. W. Kyle, and J. L. Hobson. 1954. N. A. blastomycosis; clinical forms of the disease and treatment with stilbamidine and 2-hydroxystilbamidine. Ann. Internal Med. **41**: 89-107.

Takaro, T., J. E. T. Hopkins, and J. D. Murphy. 1955. The treatment of pulmonary blastomycosis. Diseases of the Chest **28**:203-216.

Utz, J. P., D. B. Louria, N. Feder, C. W. Emmons, and N. McCullough. 1958. A report of clinical studies on the use of amphotericin B in systemic fungus disease. Antibiotics Ann. 1957-58, p. 65.

Utz, J. P., A. Treger, N. B. McCullough, and C. W. Emmons. 1959. Amphotericin B; intravenous use in 21 patients with systemic disease. Antibiotics Ann. 1958-59, p. 628-634.

Weinberg, B. J., C. H. Lawrence, and A. Buchholz. 1954. Systemic blastomycosis treated with 2-hydroxystilbamidine. A.M.A. Arch. Internal Med. **94**:493.

Wigger, H. 1961. A case report of N. A. blastomycosis with review of the literature. Clin. Proc. Children's Hospital, Washington, D. C. **17**:59-73.

# CHEMOTHERAPY OF NORTH AMERICAN BLASTOMYCOSIS CASES OBSERVED FROM 2 TO 10 YEARS

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Therapeutic trials carried out in the same institution by the same persons over periods of several years may have some advantages over studies based on review of the literature. Such a study of North American (NA) blastomycosis was carried out at the Veterans Administration and the John Gaston Hospitals in Memphis, during the past 12 years; 36 cases were seen, and they received 43 courses of therapy. The study yielded comparative data as to the results of three different therapeutic regimens. It also provided data for comparison with the results obtained after comparatively long follow-up periods in the same patients. To compare the results of short and long follow-up periods, follow-up data obtained in 1959 are reported for 25 cases previously reported. These cases comprise FP, BK, CC, and TH in Table 3, that were first reported by Snapper and McVey (1953), and the 21 cases in Tables 1, 2, and 3, which were first reported by Sutliff, Kyle, and Hobson (1954).

A summary is given in Table 1 of the results of therapy of 36 NA blastomycosis cases with nonspecific measures, aromatic diamidines,

TABLE 1. *Summary of the results of therapy of 36 NA blastomycosis cases with average follow-up of 2 to 4 years*

| Therapy | Nonspecific | Stilbamadine, hydroxystilbamadine | Amphotericin B |
|---|---|---|---|
| Average follow-up, months | 47 | 50 | 24 |
| Arrested | 4 | 18 | 4 |
| Improved | 0 | 1† | 0 |
| Unimproved | 3* | 3† | 0 |
| Died | 4 | 5 | 1 |
| Total courses of therapy | 11 | 27 | 5 |

*Subsequent therapy with stilbamadine, 1 case, or hydroxystilbamadine, 2 cases.
†Subsequent therapy with amphotericin B, 1 case.

or amphotericin B, with average follow-up periods from 2 to 4 years for each group and which ranged from 1 month of observation in a fatal case, dying during treatment, to 122 months or 10 years of observation. The nonspecific therapy group was the only one to have a majority of cases classified as unimproved or died. The aromatic diamidines group had about 60% arrested cases, the same proportion as in the literature. The proportions of favorable and unfavorable results of amphotericin B therapy were less significant because of the small number of cases. This does not indicate any lack of interest in this antibiotic. Subsequent cases in these hospitals were included in a cooperative study, and they are thus not available for publication until after the publication of the cooperative study.

Just as in the cases reported by others, a fairly prompt and regular effect of the therapy was noted on the superficial lesions of the disease but no further specific details of this effect were tabulated.

Comparisons of the condition of three groups of patients as first reported by Snapper and McVey (1953) and by Sutliff et al. (1954) after short follow-up periods were made with their status in 1959 after a longer follow-up period.

Eleven courses of nonspecific therapy, which were first reported in 1954 (after an average follow-up of 30 months per course), showed no change when followed up again after an average period of 47

TABLE 2. *North American blastomycosis cases treated before aromatic diamidines were used (average follow-up, 47 months)*

| Patient | Lesions | Therapy | Outcome | Duration before admission | Observation |
|---|---|---|---|---|---|
| | | | | *months* | *months* |
| JLH | Osteomyelitic | KI | Arrested | 13 | 22 |
| WCB | Pulmonary | Lobectomy | Arrested | 1 | 122 |
| LP | Pulmonary | Lobectomy | Arrested | 36 | 82 |
| GB | Cutaneous | Excision | Arrested | 24 | 85 |
| JFR | Pulmonary, cutaneous | KI | Arrested, died cause unknown | 1.5 | 30 |
| HEB* | Cutaneous (3 sites) | Various | Unimproved | 8 | 50 |
| HMG* | Cutaneous | KI | Unimproved | 8 | 14 |
| ST† | Pulmonary, laryngeal, cutaneous | Various | Unimproved | 29 | 23 |
| FT | Pulmonary, osteomyelitic | Pneumonectomy excisions | Died | 24 | 42 |
| EL | Cutaneous (4 sites), pulmonary, generalized, terminally | Various | Died | 5 | 48 |
| JEMcK | Pulmonary, cutaneous, CNS | Various | Died | 2.5 | 5 |

*Treated without diamidines and later with stilbamidine. See Table 2.

†Treated without diamidines and later with stilbamidine and 2-hydroxystilbamidine. See Tables 2 and 3.

months per course of therapy. The nonspecific therapy consisted of one or more of the following: potassium iodide (saturated solution), surgery, and antibacterial chemotherapy. Only one living patient, who had not had subsequent therapy, was not available for this follow-up. It was concluded that the original observation, for an average of 30 months per course of therapy, was sufficient to yield a permanently valid result in this group of cases.

Seven cases treated with stilbamidine, and first reported after an average follow-up of 16.4 months per case, also showed no further changes when examined after an average follow-up period of 48 months per course of therapy. Again, only one living patient was not available for follow-up.

TABLE 3. *North American blastomycosis cases treated with stilbamidine (average follow-up, 48 months)*

| Patient | Lesions | Total dose | Results | Duration before admission | Observation (months) | | |
|---|---|---|---|---|---|---|---|
| | | | | | Before therapy | During therapy | After therapy |
| | | *g* | | *months* | | | |
| *HEB** | Cutaneous (3 sites) | 3.82 | Arrested | 8 | 50 | 5 | 72 |
| CEC | Pulmonary, cutaneous (5 sites), osteomyelitic | 4.05 | Arrested | 1 | 11 | 2-1/2 | 103 |
| PE | Pulmonary, cutaneous | 3.75 | Arrested | 2 | 6 | 2 | 99 |
| CLC† | Pulmonary, cutaneous | 1.65 | Arrested | 1 | 3 | 1/3 | 102 |
| *HMG** | Cutaneous | 5.75 | Arrested (died, heart disease) | 8 | 6 | 4 | 9 |
| MG | Pulmonary, GU | 3.0 | Arrested | 3 | 1-1/2 | 1-1/2 | 19 |
| *ST*‡ | Pulmonary, laryngeal, cutaneous | 8.2 | Unimproved | 29 | 23 | 4-1/2 | 11-1/2 |

*Treated without diamidines and with stilbamidine. See Table 1.

†Treated with stilbamidine and with 2-hydroxystilbamidine. See Table 3.

‡Treated without diamidines and with stilbamidine and 2-hydroxystilbamidine. See Tables 1 and 3.

Twelve courses of therapy with hydroxystilbamidine first reported after an average follow-up of 11 months per case were observed again after an average follow-up of 59 months per case. A total of 7 of these 12 cases progressed or regressed further after the first report. It was apparent that an average of 11 months follow-up per case was in this instance too short to record the final stable result. Here again only one patient was not available for follow-up.

TABLE 4. *North American blastomycosis cases treated with 2-hydroxystilbamidine (average follow-up, 59 months)*

| Patient | Lesion | Total dose | Result | Duration before admission | Observation (months) | | |
|---|---|---|---|---|---|---|---|
| | | | | | Before therapy | During therapy | After therapy |
| | | *g* | | *months* | | | |
| FP | Pulmonary, cutaneous (3 sites), GU | 5.39 | Died | 7 | 1 | 1 | 52 |
| BK | Cutaneous (2 sites) | 5.50 | Arrested | 12 | 1/2 | 1 | 13 |
| CC | Cutaneous | 4.5 | Arrested | 84 | 1/2 | 1 | 24 |
| TH | Osteo, pulmonary, generalized | 4.35 | Arrested | 7 | 0 | 2 | 85 |
| FA | Pulmonary, cutaneous, generalized | 8.1 | Arrested | 3 | 1 | 2 | 23 |
| CLC* | Pulmonary, cutaneous | 3.0 | Arrested | 1 | 5 | 1 | 90 |
| OGT | Pulmonary | 20.0 | Arrested | 2-1/2 | 4 | 6-1/2 | 82 |
| HH | Pulmonary | 45.8 | Arrested | 7 | 1-1/1 | 20 | 72 |
| ST† | Pulmonary, laryngeal, cutaneous | 7.6 | Arrested | 29 | 39 | 16 | 67 |
| CH | Osteo, cutaneous | 55.6 | Unimproved | 0 | 1 | 37 | 8 |
| LR | Pulmonary | 61.1 | Unimproved | 0 | 7 | 49 | 13 |
| SB | Cutaneous, pulmonary | 45.2 | Unimproved | 41 | 74 | 36 | 3 |

*Treated with stilbamidine and 2-hydroxystilbamidine. See Table 2.

†Treated without diamidines and with stilbamidine and 2-hydroxystilbamidine. See Tables 1 and 2.

Table 5 shows both the unfavorable and favorable changes, together with further therapy administered to the seven cases in which further changes occurred. Table 6 shows the net result of the changes noted in the hydroxystilbamidine-treated cases after a longer follow-up period. Unfavorable changes consisted of two additional deaths and two additional unimproved cases. Favorable changes consisted of three cases classified as arrested. The net effect, however, was an increase of one death and a decrease of two cases classified as improved, and an increase of one arrested case. From this experience it is not possible to visualize any drastic revisions in data resulting from more adequate follow-up periods, but the need for observation periods greater than an average of 11 months is apparent because of the risk of more drastic changes. Since it required from 6 to 16 to 20 months, respectively, for the favorable cases to complete therapy, and since it required 4 or 5 years for unfavorable cases to terminate, a follow-up period of 2 to 5 years would be desirable for dependable results, using a therapeutic agent

of the potency of the aromatic diamidines in a disease of the chronicity of NA blastomycosis.

TABLE 5. *Changes in outcome of North American blastomycosis cases treated with hydroxystilbamidine disclosed by additional follow-up*

| Patient | Year of report | Lesions | Dose | Result | Duration before admission | Observation (months) | | |
|---|---|---|---|---|---|---|---|---|
| | | | | | | Before therapy | During therapy | After therapy |
| | | | | | *months* | | | |
| CH | 1954 | Cut. Pul. Ost. | 13.7 | Arrested | 0 | 1 | 4 | 1 |
| | 1961 | Cut. Pul. Ost. | *55.6* | *Unimpr.** | 0 | 1 | 37 | 8 |
| FP | 1954 | Pul. cut. | 5.4 | Arrested | 7 | 1 | 1 | 2 |
| | 1961 | Pul. cut. *GU* | 5.4 | *Died* | 7 | 1 | 1 | *52* |
| LR | 1954 | Pul. | 17.8 | Impr. | 0 | 7 | 12 | 0 |
| | 1961 | Pul. *cut. CNS* | *61.1* | *Unimpr.*† | 0 | 7 | *64* | *4* |
| SB | 1954 | Cut. Pul. | 45.2 | Unimpr. | 41 | 74 | 13 | 3 |
| | 1961 | Cut. Pul. | *80.0* | *Died* | 41 | 74 | *36* | *3* |
| OGT | 1954 | Pul. | 11.9 | Impr. | 2-1/2 | 4 | 3 | 3-1/2 |
| | 1961 | Pul. | *20.0* | *Arrested* | 2-1/2 | 4 | 6-1/2 | *82* |
| HH | 1954 | Pul. | 5.5 | Unimpr. | 7 | 1-1/2 | 1 | 1-1/2 |
| | 1961 | Pul. | *45.8* | *Arrested* | 7 | 1-1/2 | *20* | *62* |
| ST | 1954 | Cut. Laryng. Pul | 2.0 | Unimpr. | 29 | 39 | 1/3 | 12 |
| | 1961 | Cut. Laryng. Pul | 7.6 | *Arrested* | 29 | 39 | *16* | *67* |

*Treated with amphotericin B. Arrested.

†Treated with amphotericin B. Died.

TABLE 6. *Change in outcome of 12 NA blastomycosis cases treated with hydroxystilbamidine disclosed by additional follow-up*

| | 1954 Report | 1961 Report |
|---|---|---|
| Average follow-up, months | 11 | 59 |
| Arrested | 7 | 8 |
| Improved | 2 | 0 |
| Unimproved | 3 | 3 |
| Died | 0 | 1 |

Further cases of NA blastomycosis treated with hydroxystilbamidine (Table 7) and amphotericin B (Table 8) that were summarized are listed individually in the Tables. The follow-up periods were not sufficient for final results in improved or unimproved classifications, especially the total number of fatalities, but were longer than most in the literature.

Eight previously unreported cases of NA blastomycosis treated with hydroxystilbamidine are listed in Table 7, with an average follow-up of 38 months per case. The dose of hydroxystilbamidine was increased to an average of 20 g per case. Five were arrested and

three died. These results were no better than in other groups receiving stilbamidine and hydroxystilbamidine in smaller average doses.

TABLE 7. *North American blastomycosis cases treated with hydroxystilbamidine (average follow-up, 38 months)*

| Patient | Lesion | Total dose | Result | Duration before admission | Observation (months) | | |
|---|---|---|---|---|---|---|---|
| | | | | | Before therapy | During therapy | After therapy |
| | | *g* | | *months* | | | |
| EC | Cutaneous, pulmonary | 18.6 | Arrested | 3 | 1 | 3-1/2 | 76 |
| RA | Cutaneous | 10.8 | Arrested | 4 | 1/3 | 2-3/4 | 49 |
| AW | Cutaneous, pulmonary | 5.6 | Arrested | 3 | 1 | 1 | 27 |
| PP | Pulmonary | 32.1 | Arrested* | 0 | 1-2/3 | 13 | 3-1/2 |
| SD | Osteo | 8.8 | Arrested* | 6 | 3-1/2 | 7 | 38 |
| JD | Cutaneous | 21.6 | Arrested, died (surg) cause unknown | 4 | 1/3 | 13 | 59 |
| SE | Generalized | 7.0 | Died | 12 | 2/3 | 1 | 0 |
| RW | Pulmonary, cutaneous, CNS | 46.2 | Died | 12 | 3/4 | 11 | 0 |

*Died; abdominal operation and cardiopulmonary disease respectively. Postmortem showed no NA blastomycosis.

The results of five courses of amphotericin B therapy are given in Table 8. One of these courses was followed by death due to a chronic caseous central nervous system lesion; four became arrested after an average observation period of 24 months per case. Two of

TABLE 8. *North American blastomycosis cases treated with amphotericin B (average follow-up, 24 months)*

| Patient | Lesion | Total dose | Results | Duration before admission | Observation (months) | | |
|---|---|---|---|---|---|---|---|
| | | | | | Before therapy | During therapy | After therapy |
| | | *g* | | *months* | | | |
| CH* | Cutaneous, pulmonary, osteo | 0.8 | Arrested | 0 | 46 | 1 | 26 |
| LR† | Pulmonary, cutaneous, CNS | | Died | 0 | 69 | 2-1/2 | 4 |
| EH | Pulmonary | 1.22 | Arrested | 4-1/2 | 3/4 | 1 | 15 |
| CW | Pulmonary | 1.25 | Arrested | 5 | 1-2/3 | 1 | 38 |
| RP | Cutaneous, GU Cardio. | 1.03 | Arrested | 2-1/2 | 1/4 | 1-1/3 | 32 |

*Treated with hydroxystilbamidine and amphotericin B.

†Treated with stilbamidine and amphotericin B.

these cases were unimproved after hydroxystilbamidine therapy, one recovered, and one, as mentioned above, died after amphotericin B therapy.

The observation of the cases described above has shown that (i) prompt symptomatic effects occur quite regularly after administration of aromatic diamidines and amphotericin B; (ii) the fatality rate is lower with aromatic diamidines and amphotericin B therapy than in cases reported in the literature treated without these therapeutic agents; (iii) amphotericin B will cause arrest of the disease in many cases that do not respond to aromatic diamidines; (iv) valuable experience in administration techniques, the details of absorption and excretion, and toxicity of the therapeutic agents was obtained.

Studies such as those described above were not adequate to show (i) exact data on the duration of lesions in the controls and treated cases; (ii) whether the low fatality rate was due to treatment or to other factors; (iii) whether aromatic diamidines would be effective in amphotericin B-resistant cases and how many amphotericin B-resistant cases might be expected; (iv) the optimal dose; (v) whether diamidines plus amphotericin B might be superior to either therapy alone; and (vi) the relative values of the two principal therapeutic agents.

It is apparent that the accumulated data, while suitable for the limited purpose in view when it was collected, does not furnish comparisons which would increase appreciably the exactness and the amount of information to be obtained from therapeutic trials. Such studies await opportunities to accumulate sufficient cases to carry out control studies.

## Literature Cited

Snapper, I., and L. V. McVey. 1953. The treatment of N. A. blastomycosis with 2-hydroxystilbamidine. Am. J. Med. 15:603-23.

Sutliff, W. D., J. W. Kyle, and J. L. Hobson. 1954. N. A. blastomycosis: clinical forms of the disease and treatment with stilbamidine. Ann. Internal Med. 41:89-107.

# STAPHYLOCOCCAL SKIN INFECTIONS: A LONGITUDINAL CLINICAL AND SEROLOGICAL STUDY

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The study reported here was carried out under circumstances that precluded many of the precautions that have been recommended for handling the hospital staphylococcal problem. The patients were mentally retarded, a majority was toilet-untrainable, they did not comprehend the program, and they were unable to cooperate in any direct way. The patients in the study had in common a "boil problem"; multiple scars and lesions in various stages of evolution bore mute testimony to the chronicity and severity of their complaint.

Sulfonamide and antibiotic therapy, with photographic documentation of the progress of lesions, was studied; no evidence of modification of the natural evolution of a staphylococcal skin infection was found. Accordingly, the use of antibiotics was restricted to treatment of systemic illness. Treatment of furuncles consisted of wet soaks, local antiseptics, incision and drainage, and attempts to apply dressings. A "boil clinic" was organized to treat 20 to 40 patients each week.

In this setting, with clear evidence that all that had been done over a prolonged period of time had accomplished no more than a status quo, it was decided to turn to an immunobiological approach. A voluminous literature on staphylococcal vaccines (D'Antona, 1958; Ramon et al., 1946) does little to establish a cause-and-effect relationship between vaccine and effects reported (Elek, 1959; Mudd, 1960). The nonexistence of definitive criteria may account for a seeming lack of objectivity in reporting. Further, there is a lack of longitudinal records of skin infections in the same individuals.

There has been no uniformity in the terminology of the biological materials that have been employed in the treatment of furunculosis; it is now becoming clear that the distinction between a "vaccine" and a "toxoid" is probably an artificial one. Aged cultures, which are best for toxin production and the preparation of toxoids, also contain substances produced by autolysis of the organisms. Thus,

some of the best toxoids may share some of the virtues, if any, of a bacterial (vaccine) preparation.

Some investigators favor vaccines, some favor toxoids, a combination has also been suggested (Fleming, 1939). Advocacy of these treatments has rested upon the *impression* of clinical improvement. Many physicians, in desperation, treat recurrent furunculosis with autogenous vaccines (Baird, 1955), but one has the feeling that failures are overlooked and successes used as justification for continuance of this practice.

The literature also points up a lack of correlation between the stimulation of antibodies (chiefly anti-α-hemolysin) by vaccination and the subsequent course of the patient (Fleming, 1939; Longacre, 1941). Somewhat better, but still far from definitive, is the correlation of anti-leukocidin antibodies with clinical conditions (Towers and Gladstone, 1958; Sebeck, Schubert, and Johanovsky, 1958*a,b*).

## Materials and Methods

In the face of the lack of information to guide a choice of immunizing material, a choice was made of a vaccine which is prepared by bacteriophage lysis of serotype I staphylococcus (prepared and supplied by Delmont Laboratories, Swarthmore, Pa.). The finding (Boger, Frankel, and Gavin, 1960; Boger, Gavin, and Frankel, 1961) that this vaccine stimulated measurable antibody response in *some* patients encouraged us to study this material further. This preparation contains lytic products of the cell wall, soluble cytopasmic material, negligible amounts of exotoxins, the bacteriophage itself, and culture medium. The vaccine is sterile, and no preservative was added. The preparation was standardized on the basis of its content of bacteriophage particles and lytic capacity. This vaccine was regarded as an essentially bacterial (somatic) antigen prepared in a unique way, and the content of bacteriophage particles was coincidental so far as its use as a staphylococcal vaccine was concerned. The therapeutic value of bacteriophages, categorically dismissed by some (Elek, 1959), was not at issue in this investigation.

This study was directed toward (i) a longitudinal recording of the natural course of staphylococcal skin infections (specifically furuncles) in a group of hospitalized, mentally retarded patients; (ii) a clinical evaluation of the merit of a staphylococcal vaccine; (iii) a monitoring of the course of vaccination with serological determinations of an unidentified antibody, measurable by a newly described technique (Boger et al., 1960, 1961); and (iv) an attempt to correlate serological findings with the clinical course of patients with staphylococcal infections.

## Results

*Empirically treated patients.* From the "boil clinic," 30 of the "worst patients" were selected for therapy. Treatment with the vaccine followed a fixed schedule; 0.05 ml initially and progressing at 4-day intervals to 0.1, 0.2, 0.3, 0.4, and finally, 0.5 ml, subcutaneously. If tolerated without the development of areas of redness or tenderness in excess of 2 in. in diameter, weekly injections of 0.5 ml were continued indefinitely. When a local reaction of 2 in. occurred, the dose was either repeated or lessened by one step. No systemic reactions or severe local reactions at the sites of injection were observed.

Results to be anticipated from the use of the vaccine were not discussed with nursing or attendant personnel. Approximately 4 weeks after the beginning of therapy, volunteered comments indicated that the lesions "seemed to be smaller, less red, and fewer required incision and drainage." The additional observation was made by nurses that furuncles, which would, in their opinion, have required lancing previously, were resolving without necessity of incision. These "testimonials" were belittled as being uncritical and probably an effort to be "kind to the investigators." But at the end of 8 weeks, and to a greater extent at 12 weeks, all those concerned with the project were of the opinion that benefits had accrued from the use of the vaccine. The feeling on this point was so strong that an additional 14 "problem" patients were added to the treatment series.

The severity of furunculosis in this group of patients was photographically documented before treatment began. Many of the patients had 20 to 30 active lesions, without counting innumerable follicular infections, and a majority of these patients were described as being "boil repeaters" for years.

After 6 months, this group of treated patients was so much improved that skeptics in other portions of the hospital who had patients with chronic furunculosis asked to have some of their patients included in the project. The natural course of furuncles is admittedly variable, and there is no way to isolate the effects of the vaccine from the beneficial effects of heightened enthusiasm on the part of those who saw something "being done for patients." However, there was a strong feeling that this group of patients had benefited. An attempt to "objectively" evaluate our observations by reviewing the records was disheartening, for it was discovered that there were no observations about the sizes of the lesions. Mere numbers of lesions failed to reflect the very striking differences in the size and character of the lesions that were noted as treatment progressed. Only the colored photographs document the real severity of the preexistent circumstances.

*Second study series.* During the treatment of the foregoing series, another group of comparable but untreated male patients

continued to present chronic furunculosis. The sex of the two groups was different, but hospital conditions and seasons of the year were the same.

Accordingly, a group of 23 male patients was selected for study. Recording of numbers *and* size of furuncles were begun. All patients were selected because of having a "problem," but the "problem" was not clear from the record of patients 11, 12, 13, and 14 during the 4 months prior to treatment. Active vaccination was begun on 12/8/59 and continued for approximately 14 months (Table 1). Patients 13 and 14, selected because of a supposed "problem," failed to show any evidence of furunculosis during the entire period of observation. Such findings cast doubt on some clinical "impressions." Patient 2, a flagrant example of recurrent abscesses, represents a complete failure of treatment.

Once again the clinical impression was one of benefit from treatment. Experience indicated that 4 to 8 weeks was required for the effects of vaccination to become apparent; Table 1 suggests that there may have been an over-all improvement at this time. However, there was no control group nor was there a long period of observation prior to treatment. This defect was emphasized by patients 13 and 14.

On 2/21/61 this group of patients was randomly separated into treatment and control groups. Subsequent to that date and up to 10/10/61, each patient received a weekly injection; half continued to receive active vaccine (patients 1, 3, 6, 7, 9, 10, 14, 15, 17, 20, 22, and 23), and the others were injected with a placebo. Furuncle incidence during the period of observation was so low that no difference between the groups could be established. Whether difference in therapy was being tested under these conditions can be called into question, since previous work has shown that elevated antihemolysin titers may persist for as long as 1 year after discontinuance of vaccination (Fleming, 1939). Thus a group judged to be a "control group" by medication criteria may fail to be one by laboratory criteria.

Patient 2, who had one of the worst records of recurrent furunculosis, seemed actually to improve in months four and five of 1961 after having been put on placebo. In 1960, the patient was similarly free of furuncles during the fourth and fifth months. This patient is one of the best examples of what appears to be a "cycling" of skin infections from one year to another. This puzzling phenomenon has occurred in other patients. A sequence such as this can contribute to erroneous judgments about the success of a treatment or it can serve as an excellent guide to effectiveness of a treatment if the patient is followed long enough and the sequence is modified.

This second series of patients was studied with much more care than the original empirically treated group, but it was not divided randomly into treatment and placebo groups at the time of its original

TABLE 1. *Longitudinal study of 23 male patients*

| | 1959 | | | | 1960 | | | | | | | | | | | | 1961 | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Patient | 9 | 10 | 11 | 12* | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 1 | 2† | 3 | 4 | 5 | 6 | 7 | 8 | 9 |
| 1 | | | $L^{1}$‡ | | | $L^{1}$ | $L^{1}$ | $L^{1}$ | | | | $S^{1}$ | $L^{1}S^{3}$ | $L^{1}$ | $L^{1}$ | | | | | | | | | | |
| 2 | $S^{3}$ | | | | $L^{2}$ | $L^{3}$ | $L^{3}$ | | | $S^{7}$ | | | $L^{8}S^{1}$ | $L^{1}S^{3}$ | $L^{6}$ | | $L^{1}S^{2}$ | $L^{5}$ | $L^{1}$ | | | $S^{1}$ | | | $L^{1}S^{1}$ |
| 3 | $S^{2}$ | | | | $S^{2}$ | $S^{3}$ | $S^{3}$ | | $L^{2}$ | | | | | | | | | | | $S^{1}$ | $S^{1}$ | $S^{1}$ | $S^{1}$ | | $S^{1}$ |
| 4 | $S^{2}$ | $S^{1}$ | $S^{1}$ | | $L^{1}S^{3}$ | $L^{1}$ | $L^{1}$ | | $L^{1}$ | $S^{1}$ | | $S^{1}$ | | | | | $L^{1}$ | | $L^{1}$ | | | | | | |
| 5 | $S^{2}$ | $S^{3}$ | | | | | $S^{1}$ | | | | | | | | | | | | | | | | | | |
| 6 | | | $S^{1}$ | | | | | | | | | | | | | | | | | | | | | | |
| 7 | $S^{1}$ | | | $S^{1}$ | $S^{1}$ | | | | | | | $S^{1}$ | | | | | | | | | | | | $S^{1}$ | |
| 8 | | | $S^{2}$ | $L^{1}$ | | $L^{2}$ | | $S^{1}$ | $L^{2}$ | | | $S^{2}$ | | | | | | | | $S^{3}$ | $L^{1}$ | $L^{1}$ | | | |
| 9 | $S^{1}$ | | $L^{1}$ | $S^{1}$ | | | | $S^{1}$ | | | | $L^{1}$ | | | | $L^{1}$ | $S^{1}$ | $S^{1}$ | | $S^{1}$ | $S^{1}$ | $S^{1}$† | | | |
| 10 | $L^{1}$ | $L^{1}S^{1}$ | $L^{2}S^{1}$ | $S^{6}$ | $S^{3}$ | $S^{1}$ | $L^{2}$ | $L^{2}S^{3}$ | $S^{3}$ | $S^{1}$ | $S^{2}$ | | | | | | | $S^{1}$ | | | | | $S^{1}$ | | |
| 11 | | | | | | $S^{2}$ | | | | | | | | | | | | | | | | | | | |
| 12 | | | | | $L^{8}$ | | | | | | | | | | | | | | | | | | | $S^{1}$ | $S^{1}$ |
| 13 | | | | | | | | | | | | | | | | | | | | | | | | | |
| 14 | | | | | | | | | | | | | | | | | | | | | | | | | |
| 15 | $S^{2}$ | $S^{1}$ | $S^{2}$ | | | $S^{1}$ | | $S^{1}$ | | | $S^{1}$ | | $S^{1}$ | | | | | | | | | | | | |
| 16 | | | $L^{1}$ | | | | | | | | | | | | | | | | | | | | | | |
| 17 | | | $L^{1}$ | | | | | | | | | | | | | | | | | | | | | | |
| 18 | | | $S^{1}$ | | | $S^{1}$ | | | | | | | | | | | | | | | | | | | |
| 19 | $S^{1}$ | | | | | | | | | | | | | | | | | | | | | | | | |
| 20 | $S^{2}$ | | | | | | | | | | | | | | | | | | | | | | | | |
| 21 | $L^{1}$ | | $L^{1}$ | | | | | | | | | | | | | | | | | | | | | | |
| 22 | | | $S^{2}$ | | | | | | | | | | | | | | | | | | | | | | |
| 23 | | $L^{1}$ | | | $L^{3}$ | $S^{1}$ | | | | | | | | | | | | | | | | | | | |

*December 8, 1959 - Empiric use of staphylococcus antigen lysed with bacteriophage.

†February 21, 1961 - "Blind study" begun.

‡L="large" boil, larger than 1 inch in diameter; S="small" boil, less than 1 inch in diameter.

selection; the basic motivation of the program was one of therapy and not of evaluation of any specific treatment.

*Controlled studies.* The two foregoing groups did serve to establish the pattern for an evaluation of therapy of chronic furunculosis. Since the previous groups of patients had been selected as the "worst patients," those that remained were, by definition, less acute problems. Nevertheless, two groups, 16 males and 16 females, were selected, on the basis of the best records of past infections against which to judge benefits.

Each 16 was randomly divided into treatment and control groups. Although there were marked individual differences in the numbers of infections during the months prior to the initiation of the controlled observations, these patients were the best available challenge of therapy. Beginning on 2/21/61 the patients received subcutaneous injections according to the schedule described above. The preparations were well tolerated, and it was not necessary to interrupt therapy in any patient.

The over-all impression of the observer team was that the total incidence of furuncles in the institution had been lessening, and that the acuteness of the problem had abated steadily over the years of study. Whether this change has been coincidental, or whether it was in some way related to the heavy vaccination program of the "most difficult patients" and the general heightened interest in the problem, cannot be determined. As the observation of the last two groups progressed (Tables 2 and 3), it was apparent that the density of infections was so low that it would be impossible to evaluate the difference between groups. However, since the study was begun during the winter and some voice the impression that furuncles are more numerous during the summer, observations were continued.

An analysis of the occurrence of furuncles by month in our observed population is shown in Table 4; there seems to be no marked seasonal difference.

Ten months of treatment with vaccine in 16 of 32 patients showed no evidence of difference between the vaccinated and placebo groups. Although the observation was not general, the occurrence of boils during the same months in the same patient over several years is interesting. Further, the sequence of freedom from skin infections for periods of several months, without any relationship to treatment, appears rather frequently in these longitudinal records. Such findings emphasize the dictum that unless a patient has been rendered free of skin infections for at least 6 months no therapeutic claim can be made. Alternating recurrence (patients 2, 8, and 15 in Table 1) represents the type of evidence used to support the concept that a patient "desensitizes" himself for a time and then infections recur when the sensitivity to staphylococcal substance has returned. This idea has been put forward by a number of workers (D'Antona, 1958; Johanovsky, 1958*a*,*b*; Wagner, 1950).

TABLE 2. *Longitudinal observation of furunculosis**

Series I males - placebo

| | | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 59 | | | | | | o | | | | | o | |
| I | 60 | o | | | x | xx | x | | | | | | oo |
| | 61 | o | | | | | | | | | | | |
| | 59 | | | | | | | | | ooo | | | |
| J | 60 | | | | | | | | xoo | | xo | xo | |
| | 61 | | oo | o | | | | | | | | | |
| | 59 | | | | | | | | | | | | |
| K | 60 | | | | oo | o | oo | | | | | xo | xxoo |
| | 61 | xo | | o | | | | | | | | | |
| | 59 | | | | | xo | oo | | x | xoo | | | |
| L | 60 | x | x | xo | x | x | x | | x | | | | o |
| | 61 | | o | x | x | x | x | o | | | | | |
| | 59 | | | | | | | | | o | | | |
| M | 60 | | o | o | o | | | | | | | | |
| | 61 | | | | | | | | | | | | |
| | 59 | | | | | | | | | | | | |
| N | 60 | | | o | | | | | | | | | |
| | 61 | | | | | | | | | | | | |
| | 59 | | | | | | | | | | | | |
| O | 60 | | x | | | | | | | | | | |
| | 61 | | | | | | | | | | | | |
| | 59 | | | | | | | | | | | | |
| P | 60 | | | | | | | | | | | | |
| | 61 | | | | | | | | | | | | |

Series I males - treatment

| | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 59 | | | | | | | | | | | | |
| A | 60 | | | | | | o | o | o | oooo | xxxxoo | | |
| | 61 | o | | | | | | | | | | | |
| | 59 | | | | | | | | | | | | |
| B | 60 | | | o | | | | | | | | o | |
| | 61 | | | | | oo | | ooo | | | | | |
| | 59 | | | | | | | | | | | | |
| C | 60 | | | | | | | | | | | x | x |
| | 61 | | o | | | | | | x | | | | |
| | 59 | | | | | | | | | | | | |
| D | 60 | o | | o | | | | | | | | | |
| | 61 | | | | x | | | | | | | | |
| | 59 | | | | | | | | | | | | |
| E | 60 | | | o | | | o | | | | | | |
| | 61 | | | | | | | | | | | | |
| | 59 | | | | | | | o | o | x | x | | |
| F | 60 | | | | | | | | | | | | oo |
| | 61 | | | | | | | | | | | | |
| | 59 | | | | | | | | | | | | |
| G | 60 | | | x | x | | | | | | | | |
| | 61 | | | | | | | | | | | | |
| | 59 | | | | | | | | | | | | |
| H | 60 | | x | o | o | o | x | x | o | | | | |
| | 61 | | | o | | | | | oo | | | | |

*x indicates furuncle larger than 1 inch in diameter; o indicates furuncle smaller than 1 inch in diameter.

*Serological studies.* Toxoids and vaccines have been standardized in terms of their content of α-hemolysin, and efficacy has been expressed in terms of their ability to stimulate the formation of anti-α-hemolysin antibodies. However, there is general agreement that there has been poor, if any, correlation between rises in antibody

TABLE 3. *Longitudinal observation of furunculosis**

Series II females - placebo

| | | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 59 | | | | | | | | x | x | | | |
| I | 60 | | | | | | | | x | x | o | | |
| | 61 | | | | | | | | | | | | |
| | 59 | | | | | | | x | | | oo | | |
| II | 60 | | | | | | | o | | o | o | | |
| | 61 | | | | | | | o | | | | | |
| | 59 | | | | | | | | | | | | |
| III | 60 | | | | | x | x | x | | | | | |
| | 61 | x | | | | | | | | | | | |
| | 59 | | | | | x | x | | | x | | | |
| IV | 60 | x | | | | | | | | | x | x | |
| | 61 | | | | o | | | | | | | | |
| | 59 | | | | | | | | | | | | |
| V | 60 | | | | x | xx | | | | | xx | | |
| | 61 | | | | | | | x | | | | | |
| | 59 | | | | | | | | | | | | |
| VI | 60 | x | | xxx | | | | | | | | | |
| | 61 | | | x | | | | | | | | | |
| | 59 | | | | | o | ooooo | o | o | o | oo | | |
| VII | 60 | | | | | | | oo | xoo | ooo | xo | | |
| | 61 | xx | | | | | | | | | | | |
| | 59 | | | | | | | | | | | | |
| VIII | 60 | | x | x | | | | | | | | | |
| | 61 | | | | | | | | | | | | |

Series II females - treatment

| | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 59 | | | | o | | oo | o | xx | x | | | |
| IX | 60 | | o | oooo | o | | | o | o | | | | |
| | 61 | | | | | | xo | | x | | | | |
| | 59 | | | | | | o | o | | | | | |
| X | 60 | | | | | | o | | o | | | | |
| | 61 | | | | | | | | | | | | |
| | 59 | | | | | x | o | | o | | | xoo | |
| XI | 60 | | o | | | o | o | | o | | | o | |
| | 61 | | | xo | | oxoo | o | | xo | | | | |
| | 59 | | | | | | | | | xx | | | xo |
| XII | 60 | | | | | | | | | | x | | x |
| | 61 | x | | | | | | | | | | | |
| | 59 | | | | | x | | | | | x | | |
| XIII | 60 | | | | | | | | | | x | x | |
| | 61 | | | | | | | | | | | | |
| | 59 | | | | | o | o | o | x | o | | | |
| XIV | 60 | | o | | x | o | oo | ooo | x | o | | o | |
| | 61 | | | | x | xoooo | o | xo | o | o | | | |
| | | | | | | ooooo | | | | | | | |
| | 59 | | | | | o | o | ooo | xx | x | o | | |
| XV | 60 | | xo | | | | | | | x | x | | |
| | 61 | x | | o | | | | | | | | | |
| | 59 | | | | | | x | xx | | | | x | |
| XVI | 60 | | | | x | | | | | | | | |
| | 61 | | | | o | | | | | | | | |

*x indicates furuncle larger than 1 inch in diameter; o indicates furuncle smaller than 1 inch in diameter.

titers and immunity. Indeed, furuncles occur despite the presence of high antihemolysin titers (Fleming, 1939; Longacre, 1941). A better correlation has been suggested for antileukocidin titers (Towers and Gladstone, 1958; Sebeck et al., 1958*a,b*).

TABLE 4. *Incidence of furuncles by month in 67 patients followed for 24 months**

| Month | No. of furuncles |
|---|---|
| Jan. | 45 |
| Feb. | 56 |
| Mar. | 52 |
| Apr. | 37 |
| May | 57 |
| June | 51 |
| July | 30 |
| Aug. | 32 |
| Sept. | 59 |
| Oct. | 36 |
| Nov. | 41 |
| Dec. | 25 |

*Range: 30 to 59; average: 43.

In a previous publication (Boger et al., 1960), a test was described which apparently measures an antibody against an as yet undefined staphylococcal factor. Variable titers of the antibody occurred in the serum of humans and also in the sera of many animal species. In direct ratio to the concentration of pooled $\gamma$-globulin, as compared to individual human sera, increased amounts of antibody were found. After vaccination with a bacteriophage-lysed vaccine, approximately 60% of the patients showed a significant rise in antibody titer. Therefore, it seemed purposeful to follow the titers of this antibody in vaccinated patients over a period of months. Serial titrations of specimens taken from the group of 23 patients (second study series) are presented in Table 5. Pretreatment titration reciprocals (column 1) range from 400 to 1600, and fall within the range of normal. It is clear that 11 of 23 patients had at least a fourfold increase in titer in response to vaccination (column 2). Vaccination at weekly intervals was continued for a year, and serum samples were obtained at intervals. Titers elevated by the vaccination remained so; a few patients failed to respond. The differences in individual responses are clear, and if a rise in titer does occur it tends to be maximal. These findings are in conformity with the work of Fleming (1939).

We interpret the rise in titer after vaccination as reflective of a response to the vaccination rather than to the existence of furuncles; these patients had been selected because of an antecedent history of furuncles and yet many of the initial titers were low. Patient 10 is a case in point; the titer was originally 1:400; after vaccination it rose to 1:3200. Patients 13 and 14, who had no abscesses in the 4 months prior to vaccination, showed an eightfold increase in titer after vaccination. Patient 12, who had no previous furuncles, had a rather low titer which rose promptly. It might be interpreted

that this rise was due to the crop of large boils which occurred approximately at the same time as the first postvaccination serum was drawn, but patient 11 showed a greater rule between the pre- and post-vaccination serum and yet had no infection. Patient 2, who showed the highest continuous titers, was also the patient who had the greatest number of active lesions over a prolonged period; this might suggest correlation of the measured titers with the clinical course.

TABLE 5. *Serological studies (Boger-Frankel-Gavin Test) in patients with chronic furunculosis*

| | 1959 | 1960 | | | 1961 | | |
|---|---|---|---|---|---|---|---|
| Patient | 11/8 | 1/10 | 7/6 | 10/19 | 2/8 | 4/5 | |
| 1 | – | – | 6,400 | 12,800 | 3,200 | (3,200) | 6,400 |
| 2 | 1,600 | 6,400 | 12,800 | 12,800 | 12,800 | (12,800) | 6,400 |
| 3 | 1,600 | 1,600 | 3,200 | 1,600 | 3,200 | (3,200) | 1,600 |
| 4 | 800 | 6,400 | 3,200 | 6,400 | 3,200 | (3,200) | 6,400 |
| 5 | 1,600 | 3,200 | 6,400 | 3,200 | 3,200 | (3,200) | 3,200 |
| 6 | 800 | 3,200 | 3,200 | 3,200 | – | – | – |
| 7 | 800 | 800 | 3,200 | 1,600 | 1,600 | (3,200) | 1,600 |
| 8 | 1,600 | – | 3,200 | 1,600 | 1,600 | (1,600) | 3,200 |
| 9 | 800 | 800 | 3,200 | 3,200 | 3,200 | (3,200) | 1,600 |
| 10 | 400 | 3,200 | 3,200 | 3,200 | 1,600 | (1,600) | 3,200 |
| 11 | 800 | 6,400 | 6,400 | 3,200 | 3,200 | (3,200) | 1,600 |
| 12 | 800 | 3,200 | 3,200 | 1,600 | 3,200 | (3,200) | 6,400 |
| 13 | 400 | 3,200 | 3,200 | 1,600 | 1,600 | (3,200) | 3,200 |
| 14 | 400 | – | – | 3,200 | 1,600 | (1,600) | 1,600 |
| 15 | 800 | 6,400 | 12,800 | 1,600 | 3,200 | (1,600) | 1,600 |
| 16 | 400 | 1,600 | – | 1,600 | 3,200 | (3,200) | 3,200 |
| 17 | 1,600 | 1,600 | 3,200 | 800 | 6,400 | (6,400) | 6,400 |
| 18 | 1,600 | 1,600 | 1,600 | 1,600 | 3,200 | (6,400) | 3,200 |
| 19 | 1,600 | 3,200 | 1,600 | 1,600 | 3,200 | (1,600) | 1,600 |
| 20 | 400 | 1,600 | 1,600 | 6,400 | 800 | (1,600) | 1,600 |
| 21 | 800 | 1,600 | 3,200 | 1,600 | 1,600 | (3,200) | 3,200 |
| 22 | 800 | 1,600 | 1,600 | 1,600 | 800 | (1,600) | 1,600 |
| 23 | 400 | 1,600 | 3,200 | 1,600 | 1,600 | (3,200) | 3,200 |

*Multiple antigens.* Any coagulase-positive staphylococcal strain can be employed in the staphylococcal agglutination test, but strains differ in their sensitivity (Boger et al., 1960). An antibiotic-resistant, reactive strain was selected as a standard and has been continuously employed in our laboratory.

Although the bulk of our studies has been done using this standard strain, it had occurred to us that the differences in titer observed in the same serum when different staphylococcal strains were employed might have significance. Thirteen strains which Oeding (1960) has employed for this factor analysis of staphylococci were used as antigens in the agglutination test to titrate three pairs of pre- and postvaccination sera (Table 6). Despite the serological similarity of

Standard #7 and the Cowan I strain, the former is a more sensitive indicator in the agglutination test. Further, the vaccine prepared by the bacteriophage lysis of a *single* type I strain of *Staphylococcus* stimulated an elevation of antibodies to a number of the Oeding strains.

TABLE 6. *Spectrum of staphylococcal antibodies in pre-and postvaccination sera from patients vaccinated with staphylococcal vaccine*

| Antigens (Oeding strains) | Staphylococcus agglutinin titer (reciprocal) | | | | | |
|---|---|---|---|---|---|---|
| | WT (11) | | KG (16) | | M Mc (15) | |
| | pre 11/25/59 | post 1/7/60 | pre 11/25/59 | post 1/7/60 | pre 11/25/59 | post 1/7/60 |
| Wood 46 | 1,280 | 2,560 | 320 | 320 | 160 | 320 |
| F 21 | 160 | 2,560 | 320 | 1,280 | 80 | 320 |
| Cowan I | 2,560 | 5,120 | 160 | 320 | 80 | 160 |
| Cowan II | 640 | 640 | 320 | 320 | 160 | 320 |
| Cowan III | 320 | 5,120 | 80 | 1,280 | 160 | 640 |
| S-2095 | 2,560 | 5,120 | 160 | 320 | 40 | 80 |
| S-2253 | 80 | 10,240 | 80 | 1,280 | 80 | 320 |
| S-3647 | 320 | 10,240 | 160 | 1,280 | 80 | 160 |
| S-17A* | – | – | – | – | – | – |
| S-28 | 20 | 10,240 | 40 | 320 | 20 | 160 |
| S-1503 | 80 | 2,560 | 80 | 640 | 40 | 160 |
| S-3189 | 160 | 10,240 | 80 | 320 | 80 | 1,280 |
| S-365 | 320 | 20,480 | 80 | 1,280 | 40 | 80 |
| Std # 7 | 640 | 5,120 | 320 | 2,560 | 320 | 1,280 |

*Culture S-17A gave a diffuse type of growth that did not give an end point in test.

## Discussion

The circumstances of urgency under which this program of study was initiated made it nearly impossible to set up satisfactory controls. The group of 44 patients studied represented a "staphylococcal problem" and all usual therapeutic measures were conceded to have failed before a patient was accepted into the treatment group. Nevertheless, after an intensive vaccination program resulting in general agreement that the patients had benefited, there is no satisfactory answer for those who declare that such events might have occurred spontaneously. Numbers of lesions per patient were so impressive that earnest efforts had been made to make a daily record of the presence or absence of furuncles in the group under study. Failure to record particulars as to size of lesion resulted in an incomplete picture of the improvement.

When a patient has numerous active lesions of varying size and at various stages of evolution, it is a major undertaking to enumerate them, a nearly insurmountable task to make an accurate day-to-

day record of the course of these lesions, and an impossible job when the patient has "pimples" or follicular infections that may number a hundred or more. This problem was not infrequent, especially when the purulent secretions from some of the draining abscesses set up satellite situations in many of our patients. One investigator has been bold enough to mention "200 lesions" on a single patient (McCoy and Kennedy, 1960). "Inaccuracies" are inherent in the records of staphylococcal skin infections and the tables presented in this paper are our most "reasonable compromise with reality."

At this point it might be appropriate to say that the terms furuncle and furunculosis may be approved technical nomenclature, but as vehicles of communication they fall far short of the homely terms "pimple," "boil," and "carbuncle." These words have a connotation of size and character of lesion that is rather generally understood, but the term furuncle has no similar differential connotation or descriptive value. Most of the patients in this study had boils frequently complicated by carbuncles.

We were prepared to discover a seasonal relationship in the frequency of occurrence of staphylococcal skin infections, but the actual record in our study group failed to sustain any concept of seasonal incidence. This statement should not be interpreted as being in conflict with the experience of others who have documented their findings, but rather as a statement limited to conditions of this study, i.e., large day halls, frequently overheated in the winter.

Despite the lack of "controls" in the original, empirically treated group of 44 female patients with chronic furunculosis, the fact was that a quite similar group of male patients who were not vaccinated continued, during the same period of time, to have recurrent staphylococcal skin lesions. Whether it was passage of time or mere chance, the second group of male patients, who were vaccinated, seemed to show a declining incidence of active lesions during the year following the institution of an intensive vaccination program. Certain individuals showed no observable modification in their clinical course as a result of vaccination, and these cases can be regarded as distinct failures of the treatment.

Those vaccines which have shown stimulation of antibodies by the agglutination test have all contained somatic components, as suggested by the method of preparation. Of some interest is the fact that some lots of the bacteriophage-lysed vaccine and certain commercially available toxoids have not given significant rises of antibody titers as measured by the Boger-Frankel-Gavin test.

The serial titrations of antibodies in the groups of furuncle patients who have been intensively vaccinated indicates (i) marked individual responses, (ii) maximal response, if any, and reasonable maintenance of antibodies at this level, and (iii) an apparent lack of relationship of the antibody titers (using Standard #7 in the test) with the clinical course of staphylococcal skin disease. New observations

give indication that the use of *multiple* staphylococcal strains as antigens in the test system may give a better understanding of immunological response to staphylococcal vaccines.

The use of 13 strains standardized by Oeding for his factor analysis of staphylococci, as antigens in the Boger-Frankel-Gavin test, has shown differing titers of antibodies to the different antigens, not only in prevaccination sera but also in postvaccination sera (Table 6). These observations are particularly interesting since the vaccine which has been used in this study was prepared from a single strain of type I staphylococcus. The suggestion is made that an antigen or antigens are shared by many strains of staphylococci and in varying amounts.

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## Literature Cited

Baird, K. A. 1955. The myth of autogenous vaccines. Am. Practitioner and Dig. Treatment **6**:211–213.

Boger, W. P., J. W. Frankel, and J. J. Gavin. 1960. Detection and titration of *Staphylococcus aureus* agglutinin in serum. Proc. Soc. Exptl. Biol. Med. **104**:639–642

Boger, W. P., J. J. Gavin, and J. W. Frankel. 1961. A test for detection and titration of *Staphylococcus aureus* agglutinins in serum. Proc. VIIth Inter. Congr. Biol. Standardization, Aug. 27-31.

D'Antona, D. 1958. Staphylococcal toxoid in human medicine. Proc. IVth Intern. Congr. Biol. Standardization, p. 3–71.

Elek, S. D., 1959. *Staphylococcus pyogenes* and its relation to disease. Livingstone Ltd., Edinburgh.

Fleming, A. 1939. Recent advances in vaccine therapy. Brit. Med. J. **2**:99–104.

Johanovsky, J. 1958*a*. Role of hypersensitivity in experimental staphylococcal infection. Nature **182**:1454.

Johanovsky, J. 1958*b*. The importance of hypersensitivity in experimental staphylococcal infection. Ceskoslov. mikrobiol. **3**:72–81.

Longacre, A. B. 1941. Studies of antihemolysin level in patients with staphylococcus infections treated with staphylococcus toxoid. Surgery **10**:576–591.

McCoy, K. L., and E. R. Kennedy. 1960. Autogenous vaccine therapy in staphylococcic infections. J. Am. Med. Assoc. **174**:35–38.

Mudd, S. 1960. Practicability of enhancing specific resistance to staphylococcic infection. J. Am. Med. Assoc. **173**:1360–1361.

Oeding, P. 1960. Antigenic properties of *Staphylococcus aureus*. Bacteriol. Rev. **24**:374–396.

Ramon, G., R. Richou, P. Mercier, and G. Holstein. 1946. Ten years application of staphylococcal anatoxin to the therapy of infections due to the staphylococcus in human and veterinary medicine. Rev. immunol. **10**:71–81.

Sebeck, V., J. Schubert, and J. Johanovsky. 1958*a*. Possibilities of immunological prevention of staphylococcus infection of mothers and newborns. II. The influence of active and passive immunization on the morbidity rate of newborns. Casopis lékáru ceských. **97**:583-587.

Sebeck, V., J. Schubert, J. Johanovsky. 1958*b*. The possibility of immunological prevention of staphylococcal infections in mothers and newborns. III. The relationship between immunological condition and the occurrence of post-partum infections in mothers. Ceskoslov. epidemiol. mikrobiol. immunol. **7**:94-101.

Towers, A. G., and G. P. Gladstone. 1958. Two serological tests for staphylococcal infection. Lancet **2**:1192-1195.

Wagner, V. 1950. Allergy to staphylococcal anatoxin. Casopis lékáru ceských. **89**:157-159.

# KANAMYCIN IN THE SURGICAL TREATMENT OF PULMONARY TUBERCULOSIS

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During the past few years we have encountered an increasing number of patients with advanced pulmonary tuberculosis and drug-resistant organisms. A considerable number have already received not only streptomycin, *p*-aminosalicylic acid, and isoniazid, but also "secondary" drugs such as cycloserine, pyrazinamide, and viomycin. This has necessitated a continuous search for new drugs to use with surgery. One of the drugs which we have discovered to be useful is kanamycin. Since little has appeared in the literature regarding its use in surgery, our experience is herein reported.

## Materials and Methods

Between April 1958 and August 1961, 105 surgical patients received kanamycin at the Ray Brook State Tuberculosis Hospital. Two patients had minimal, 30 moderately advanced, and 73 far advanced pulmonary tuberculosis (National Tuberculosis Association classification). Two patients harbored organisms resistant to five drugs; 14, resistant to four drugs; 53, resistant to three drugs; 23, resistant to two drugs; and 10, resistant to one drug. Susceptibility studies could not be performed upon the organisms of two patients, since they did not excrete culturable *Mycobacterium tuberculosis* while at Ray Brook. One patient suspected of harboring resistant organisms was found later to have organisms susceptible to all drugs for which in vitro tests were available.

A total of 118 patients received kanamycin at the time of surgery (pulmonary resection, 94; other surgery, 24). In addition, 30 patients received preoperative kanamycin (prior to pulmonary surgery), and 8 patients received postoperative kanamycin, usually as a substitute for other drugs to which the patient had demonstrated intolerance or to which the organisms had become resistant. Several indications may have been present in the same patient.

Kanamycin was prescribed in combination with one or more other drugs to which the patients' organisms were susceptible, usually cycloserine or pyrazinamide. *p*-Aminosalicylic acid was employed

whenever organisms were susceptible to the drug. Isoniazid was not used, since organisms were invariably resistant or suspected of being resistant to this drug. Streptomycin and viomycin were not administered with kanamycin because of similar toxicity. 2,4'-Diamino-5-thiazolylphenyl sulfone (Promizole) was used until laboratory evidence indicated that blood levels were far below those required for in vitro antituberculous effect (Pecora, 1961). Amithiozone was used only when other drugs were not available.

When patients developed toxicity to one or more drugs, others were substituted with minimal delay. Drug therapy was coordinated with surgery as much as possible. A few patients received several months of preoperative therapy in an attempt to evaluate the advantages of prolonged therapy in preparation for surgery. Most of the patients received the drugs for only 2 or 3 days prior to surgery. Whenever possible, an attempt was made to treat the patient for a total period of 1 year, but it was sometimes difficult to accomplish this because of drug intolerance and poor patient cooperation. A few patients received therapy for more than 1 year, in an attempt to determine the value of prolonged therapy in the postsurgical infectious patient.

The following daily dosages were administered: amithiozone, 150 mg; cycloserine, 500 mg; kanamycin, 500 mg (four patients received 1 g daily and four received 1 g three times a week); *p*-aminosalicylic acid, 12 g; Promizole, 6 g; and pyrazinamide, 3 g. Viomycin, 1 g twice a day twice weekly, and streptomycin 1 g daily, were administered when it was necessary to substitute these drugs for kanamycin.

The following minimal laboratory tests were performed for patients receiving amithiozone: (weekly) hemoglobin, white blood count, differential and sedimentation rates; (biweekly) urinalysis and liver function tests. Patients receiving cycloserine: urinalysis (biweekly). Patients receiving kanamycin: audiogram and urinalysis (biweekly); blood urea nitrogen and sputum concentrate and culture for acid-fast bacilli (monthly). Patients receiving pyrazinamide: liver function tests and urinalysis, including test for bile (monthly). Patients receiving Promizole: hemoglobin and white blood count (weekly); urinalysis (biweekly). Patients receiving viomycin: blood urea nitrogen (monthly); urinalysis and audiograms (biweekly).

*M. tuberculosis* was considered susceptible to amithiozone, pyrazinamide, and Promizole if the host had not received them previously, since in vitro susceptibility tests were not performed for these drugs. In vitro susceptibility was determined for other drugs by inoculating organisms on (Hohn's) egg medium containing 25 $\mu$g per ml of cycloserine or viomycin, 10 $\mu$g per ml of streptomycin or *p*-aminosalicylic acid, or 1 $\mu$g per ml of isoniazid. Dubos liquid medium, containing 1.25 $\mu$g per ml of kanamycin, was also used. Direct sensitivity tests to streptomycin, *p*-aminosalicylic acid, and isoniazid were read in

6 weeks. Indirect sensitivity tests for these drugs, and all of the cycloserine and viomycin tests, were read in 4 weeks. Kanamycin tests were read in 2 weeks. Generally, any growth in the presence of a drug was considered evidence of bacterial resistance to the drug, provided that there was abundant growth in the control tube. However, viomycin and cycloserine tests were more erratic than the others, yielding occasional growth even when the patient gave no history of having previously received the drugs. For this reason, occasional growth was not considered evidence of resistance to these drugs.

For analysis, patients undergoing pulmonary resection were divided into infectious and noninfectious groups, depending upon whether or not culturable *M. tuberculosis* was recovered from the tissue or the expectorations at the time of surgery. The prognosis in these two groups differs considerably. Postoperative tuberculosis complications include failure of expectorations to become noninfectious in the absence of a contralateral source of infectious sputum, empyema, and bacteriological relapse with persistence of infectious sputum. The mere presence of viable *M. tuberculosis* in the sputum for a short period of time, followed by their disappearance without altered treatment, was not considered a complication. Early complications include those occurring within the first postoperative month; late complications include the remainder.

## Results

*Preoperative therapy.* Kanamycin, combined with other drugs, was administered preoperatively for periods in excess of a few days to 30 infectious patients. Clinically, little effect was noted except with pyrazinamide, when decrease in fever and volume of expectoration occurred. Table 1 summarizes the bacteriological results. Although the bacillary content of the expectorations decreased in some patients, sterilization was achieved in only three. Prolongation of therapy for as long as 20 months did not seem to improve results; the incidence of postoperative tuberculous complications was similar to that which occurred following only 2 or 3 days of preoperative therapy.

*Pulmonary resection.* A total of 94 pulmonary resections were performed on 81 patients. After 17 resections, performed on patients who did not harbor culturable *M. tuberculosis* at the time of surgery, no tuberculous complications followed; 77 resections, performed on patients who harbored viable organisms at the time of surgery, were followed by 11 (14%) early and 4 (5%) late tuberculous complications. Table 2 summarizes the results of pulmonary resections performed on infectious patients, according to drug regimen prescribed at the time of surgery. Of 81 patients who had pulmonary resection, 64

(79%) are noninfectious, 10 (12%) are living and infectious, and 7 (9%) are dead. Of the ten patients who are still infectious, four have a contralateral cavity. The organisms of three patients developed resistance to kanamycin. Two patients experienced severe psychotic episodes after surgery, and four others were so uncooperative as to render adequate postoperative care impossible. The principal causes of death, determined clinically or if possible by postmortem

TABLE 1. *Bacteriological results of preoperative antituberculous drug therapy**

| Duration of therapy | Number of patients | | Drug regimen | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | KCPz | | KPz | | KPa | | KC | | KPr | |
| | T | N | T | N | T | N | T | N | T | N | T | N |
| *months* | | | | | | | | | | | | |
| 1 | 4 | | | | 4 | | | | | | | |
| 2 | 7 | 2 | 1 | 1 | 2 | | | | 2 | 1 | 2 | |
| 3 | 2 | | 1 | | 1 | | | | | | | |
| 4 | 4 | | 1 | | 2 | | 1 | | | | | |
| 5 | 4 | | | | 3 | | | | | | 1 | |
| 6 | 2 | | | | 1 | | | | | | 1 | |
| 7 | 2 | 1 | 1 | | | | | | 1 | 1 | | |
| 8 | 2 | | | | 1 | | | | 1 | | | |
| 9 | 1 | | 1 | | | | | | | | | |
| 10 | 1 | | | | | | | | | | 1 | |
| 20 | 1 | | | | | | | | 1 | | | |
| Total | 30 | 3 | 5 | 1 | 14 | | 1 | | 5 | 2 | 5 | |

*Symbols used: C=cycloserine, K=kanamycin, Pa=*p*-aminosalicylic acid, Pr=Promizole, Pz=pyrazinamide, T=total, N=number of patients whose expectorations and tissue excised at surgery failed to yield culturable *M. tuberculosis*.

TABLE 2. *Pulmonary resections on infectious patients*[a]

| Drug regimen | Number of resections | Extent of disease | | | Procedures | | | | Number of tuberculous complications |
|---|---|---|---|---|---|---|---|---|---|
| | | Min. | Mod. | F.A. | P | L | S | W | |
| KC | 22 | | 8 | 14 | 4 | 12 | 1 | 5 | 3 |
| KPa | 8[b] | | 1 | 7 | 1 | 4 | | 3 | 3 |
| KPz | 26[b,c] | | 7 | 19 | 3 | 13 | 6 | 4 | 3 |
| KPr | 14 | | 4 | 10 | 3 | 4 | | 7 | 2 |
| KCPz | 5 | | 1 | 4 | | 2 | | 3 | 4 |
| KCA | 1 | | 1 | | | | 1 | | 0 |
| Total | 76[d] | | 22 | 54 | 11 | 35 | 8 | 22 | 15 |

[a]Abbreviations used: A=amithiozone, C=cycloserine, K=kanamycin, Pa=*p*-aminosalicylic acid, Pr=Promizole, Pz=pyrazinamide, Min.=minimal, Mod.=moderate, F. A.=far advanced (National Tuberculosis Association classification), P=pneumonectomy, L=lobectomy, or lobectomy and segment and/or wedge, S=segmental resection or segmental and wedge, W=wedge.

[b]One patient developed kanamycin-resistant organisms during the course of therapy.

[c]One patient harbored organisms resistant to kanamycin at the beginning of therapy.

[d]One patient died two days postoperatively and is not included in this table.

examination, are listed in Table 3. The high mortality reflects to a considerable extent the large number of patients with poor cardiorespiratory reserve.

TABLE 3. *Mortality after pulmonary resections*

| Principal cause | Time after surgery | Condition of tuberculosis at death |
|---|---|---|
| Cardiorespiratory failure | 5 weeks | Infectious |
| Cardiorespriatory failure | 3 months | Infectious |
| Cardiorespiratory failure | 5 months | Infectious |
| Cardiorespiratory failure and cerebrovascular accident | 4 months | Noninfectious |
| Coronary insufficiency | 2 days | Infectious |
| Coronary insufficiency | 8 months | Noninfectious |
| Bronchogenic carcinoma | 11 months | Infectious |

From our experience, summarized in Table 2, little can be stated regarding the comparative efficacy of the various regimens employed. Certainly the three-drug regimens do not seem to be superior to the others. Thus far, in our experience, the results with kanamycin regimens are similar to those obtained with other "secondary" drugs (Pecora, 1961).

Seven patients with far advanced pulmonary tuberculosis are of special interest, since resection was performed in an effort to check progressive disease. Several of the patients were febrile. Operations included three pneumonectomies, two lobectomies, one lobectomy plus wedge, and one segmental plus wedge. Two patients developed tuberculous complications and one of them died (cardiorespiratory failure) with tuberculosis uncontrolled. At present, six patients are noninfectious with roentgenographically stable or regressive disease.

*Other surgery.* Twenty-four procedures other than resection were performed in conjunction with kanamycin therapy. Thirteen thoracoplasties, mostly of the modified Schede type (Pecora, 1958), were performed for tuberculous pleural empyemas. Five of these patients are noninfectious and two have died. Six Monaldi procedures were performed, with success in four patients. Of three pulmonary decortications for tuberculous pleural empyema, only one has been successful. Two patients who had tuberculous infections of the subscapular space after plombage thoracoplasty were successfully treated by removal of foreign material and closed suction drainage. In this series, as in one previously reported (Pecora, 1961), surgery was almost always successful if it accomplished removal or obliteration of infected spaces.

*Postoperative treatment.* Eight patients received kanamycin postoperatively as a substitute for other drugs to which they had manifested intolerance or to which their organisms had developed or were

suspected of having developed drug resistance. Of three patients who were infectious when kanamycin was started, two subsequently became noninfectious. It is interesting that both of these patients had no demonstrable evidence of cavity at the time kanamycin treatment was started.

*Toxicity.* There was no evidence of toxicity to amithiozone, cycloserine, or Promizole in this series of patients. Pyrazinamide toxicity in the form of sulfobromophthalein sodium retention was encountered in 7 (16%) of 45 patients who received the drug. After pyrazinamide therapy was terminated, all patients did well clinically.

Of 105 patients who received kanamycin, 19 (18%) developed hearing loss severe enough to require termination of therapy. Tinnitus occurred in 5 of the 19 patients. During the observation period neither tinnitus nor audioacuity improved after cessation of kanamycin. Table 4 summarizes the incidence of toxicity to kanamycin according to duration of therapy. Two of the four patients who received 1 g per day developed toxic symptoms. The four patients who received 1 g three times a week for 2 months have not manifested evidence of toxicity. There appears to be little correlation between total dosage and appearance of toxicity.

TABLE 4. *Kanamycin toxicity related to duration of therapy*

| Period of therapy | Patients treated | Toxicity | No toxicity |
|---|---|---|---|
| *months* | | | |
| 1 | 8 | 1 | 7 |
| 2 | 7 | 1 | 6 |
| 3 | 9 | 1 | 8 |
| 4 | 6 | | 6 |
| 5 | 9 | 2 | 7 |
| 6 | 12 | 4 | 8 |
| 7 | 5 | 2 | 3 |
| 8 | 11 | 2 | 9 |
| 9 | 7 | 2 | 5 |
| 10 | 3 | | 3 |
| 11 | 5 | 1 | 4 |
| 12 | 13 | 1 | 12 |
| 13 | 3 | 1 | 2 |
| 14 | 4 | 1 | 3 |
| >16 | 3 | | 3 |
| Total | 105 | 19 | 86 |

*Emergence of bacterial resistance.* Table 5 indicates the incidence of emergence of resistant variants of M. tuberculosis in patients who remained infectious while receiving kanamycin therapy. In vitro susceptibility tests were not performed for Promizole or pyrazinamide. It is interesting to note that organisms developed resistance

TABLE 5. *Emergence of bacterial resistance*[a]

| Drug regimen | Month when resistance was first detected | | | | | | | | | | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 1 | | 2 | | 3 | | 4 | | 5 | | 6 | | 7 | | 8 | | 10 | | 11 | | 12 | | 14 | | 16 | | 19 | |
| | T | R | T | R | T | R | T | R | T | R | T | R | T | R | T | R | T | R | T | R | T | R | T | R | T | R | T | R |
| KC | 1 | | 4 | | 2 | | | | 3 | 2[b] | | | 2 | | 2[c] | | 1 | | 1 | | 1 | | 1[d] | | | | 1 | |
| KPa | 2 | | | | | | | | 1 | 1 | 1 | | | | | | | | | | | | | | | | | |
| KPr | 1 | | | | | | | | | | | | 1 | | | | 1 | | | | | | | | | | | |
| KPz | 2 | 1 | 3 | | 3 | | 4 | 1[e] | 2 | 1 | 2 | | | | | | | | | | | | | | | | | |
| KCPz | | | | | | | 2 | | 1 | | | | 2 | | | | | | | | | | | | 1 | | | |
| Total | 6 | 1 | 7 | | 5 | | 6 | 1 | 7 | 4 | 3 | | 5 | | 2 | | 2 | | 1 | | 1 | | 1 | | 1 | | 1 | |

[a]Abbreviations used: C=cycloserine, K=kanamycin, Pa=*p*-aminosalicylic acid, Pr=Promizole, Pz=pyrazinamide, T=total number of patients treated, R=number of patients whose organisms developed resistant variants.

[b]One patient had received cycloserine prior to admission. Organisms susceptible to cycloserine in vitro.

[c]One patient's organisms were resistant to cycloserine at the commencement of therapy.

[d]Variants resistant to cycloserine appeared by the sixth month. Patient had previously received cycloserine.

[e]Patient had previously received kanamycin but organisms were susceptible to the drug at the start of therapy.

to kanamycin in only 6 (13%) of the 48 patients. This is in contrast to the results reported by Wright and associates (1959), all of whose patients yielded resistant variants after receiving kanamycin alone for 120 days.

One patient who had received previous cycloserine therapy yielded organisms resistant to that drug but not to kanamycin, after receiving these drugs in combination for 6 months. Another patient failed to yield organisms resistant to kanamycin after 7 months, in spite of the fact that he also received cycloserine, to which his organisms were resistant in vitro. Three patients receiving Promizole with kanamycin failed to develop resistant organisms. It is difficult to explain these results if one assumes that cycloserine is of little value in vivo against organisms which fail to demonstrate in vitro sensitivity, or if one believes that Promizole in the dosage employed is ineffective (Pecora, 1961). It is possible, of course, that the tissue concentration of kanamycin in these patients may not have been high enough to influence the selection of resistant variants. This theory might explain the persistence of infection in at least some of the patients, and would seem to agree with the theories of Haapanen and associates (1960).

*Cross resistance with viomycin.* Steenken, Montalbine, and Thurston (1959) reported the occurrence of cross resistance between viomycin and kanamycin. To determine the incidence of cross resistance between these two drugs, the charts of all patients who had received either viomycin or kanamycin and who had suitable bacteriological examinations were reviewed. Seven patients harbored kanamycin-resistant organisms which were susceptible to viomycin. One patient harbored atypical *M. tuberculosis* resistant to both kanamycin and viomycin. One patient's organisms developed viomycin and kanamycin resistance almost simultaneously while receiving kanamycin therapy. In two patients, organisms resistant to viomycin but not to kanamycin, at the start of therapy with the latter drug, subsequently developed resistance to kanamycin. One patient harbored organisms resistant to both viomycin and kanamycin, although he gave no history of having received the latter drug. Eleven patients harbored organisms resistant to viomycin but not to kanamycin. It would appear, therefore, that cross resistance between viomycin and kanamycin does not occur frequently.

## Discussion

Our experience indicates that "secondary" drug therapy yields the best results when combined with surgery. Because of this, therapy with these drugs should be withheld until a decision regarding the possibilities surgery can be determined.

Kanamycin appears to be as effective as other "secondary" drugs in our experience. Because of the occurrence of ototoxicity, it does not appear advisable to administer more than 3 to 4 g per week. Patients receiving the drug should have frequent audiograms. Whenever possible, kanamycin should be combined with other antituberculous drugs, since there is considerable evidence that this delays the emergence of resistant bacilli.

## Literature Cited

Haapanen, J., R. Gill. W. F. Russell, and I. Kass. 1960. Retreatment of pulmonary tuberculosis. Experiences with various combinations of pyrazinamide, cycloserine, and kanamycin in patients excreting tubercle bacilli resistant to both streptomycin and isoniazid. Am. Rev. Respiratory Diseases **82**:843-852.

Pecora, D. V. 1958. The surgical treatment of chronic pleural empyema. J. Thoracic Surg. **36**: 92-101.

Pecora, D. V. 1961. Drugs other than streptomycin, para-aminosalicylic acid, and isoniazid in the surgical treatment of pulmonary tuberculosis. J. Thoracic Surg. **41**:169-177.

Steenken, W., Jr., V. Montalbine, and J. R. Thurston. 1959. The antituberculous activity of kanamycin in vitro and in the experimental animal (guinea pig). Am. Rev. Tuberc. Pulmonary Diseases **79**:66-71.

Wright, K. W., A. D. Renzetti, Jr., J. Lunn, and P. A. Bunn. 1959. Observations on the administration of kanamycin to patients with tuberculosis. Am. Rev. Tuberc. Pulmonary Diseases **79**:72-77.

# HEPATOTOXIC SIDE EFFECTS OF PROPIONYL ERYTHROMYCIN ESTER LAURYL SULFATE AND TRIACETYLOLEANDOMYCIN

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Hepatic dysfunction, with or without jaundice, due to antibiotics has been rarely reported. There are several reported instances in which jaundice has been caused by chloramphenicol (Salm, 1953) and by triacetyloleandomycin (Koch and Assay, 1958). Until I (1961) reported hepatic abnormality due to propionyl erythromycin ester lauryl sulfate and triacetyloleandomycin, both of these drugs were considered relatively free from side effects. Previously unpublished information, however, does indicate that 33 cases of jaundice had been reported due to propionyl erythromycin ester lauryl sulfate since the introduction of the drug in 1958 (Kohlstaedt, 1961).

This report presents evidence of hepatic abnormality resulting from the propionyl ester of erythromycin and from the triacetyl salt of oleandomycin in 16.2% of all patients treated with these drugs for 14 days or longer. Their etiological role was further substantiated by properly controlled "challenge" with these antibiotics after all signs of previously existing dysfunction had subsided.

## Materials and Methods

A total of 152 patients with chronic pustular dermatitis was studied. The first series included 80 patients treated with propionyl erythromycin ester lauryl sulfate and 48 patients treated with triacetyloleandomycin. These two groups have since been enlarged to include 93 patients treated with the former drug and 59 patients treated with the latter.

Both drugs were administered in doses recommended by the manufacturer (1 g daily in four divided doses). Base-line hematological and liver profile studies were done prior to the administration of the drugs, and then repeated at approximately 2-week intervals while the patients were on medication. Since none of the patients in this series was institutionalized, it was not always possible to adhere to an exact 14-day interval between laboratory studies. If hepatotoxicity became apparent, the interval between studies was

decreased. Initial laboratory studies in all of the patients in these two groups were well within normal limits. Four patients in both series on the erythromycin salt and three patients on triacetyloleandomycin were challenged with the respective drugs after all signs of hepatic dysfunction had reverted to normal values. The challenging dose, in all but one patient, was 500 mg in two divided doses. The exception was one patient who left town and continued to take the drug at the rate of 0.5 g daily for several days.

After my initial report of 12.5% hepatic dysfunction in 80 patients treated with propionyl erythromycin ester lauryl sulfate, it was revealed that 33 cases of jaundice had been reported to the manufacturer. The manufacturer also received a report of liver biopsy showing periportal infiltration with lymphocytes, a few polymorphonuclear leukocytes, and many eosinophils. There was no bile stasis. After the initial series reported (Robinson, 1961), an additional test was added to the liver profile, and the alkaline phosphatase test, which was originally done by a rough screening method, was then carefully evaluated by the Sigma process. Thirteen additional patients received propionyl erythromycin ester lauryl sulfate and 14 additional patients received triacetyloleandomycin.

## Results

Three patients receiving propionyl erythromycin ester lauryl sulfate became seriously ill, with clinical features similar to those of viral hepatitis. Two developed jaundice and were hospitalized. One did not develop jaundice, but was sufficiently sick to remain in bed for approximately 2 weeks. With the exception of these three, the detection of hepatotoxicity was as a result of either mild clinical symptoms or abnormal liver function tests.

In the first two series, the serum glutamic pyruvic transaminase (SGPT) became elevated between the 14th and 21st days of therapy. In the latter two series, the serum glutamic oxalic transaminase (SGOT) test was included in the study and became elevated whenever the SGPT was elevated. Alkaline phosphatase became elevated in only 7 of the 25 patients who developed evidence of hepatic dysfunction. In all patients, the evidence of liver abnormalities was reversible when the drug was discontinued.

In each of the challenged patients, with one exception, abnormal transaminase levels were obtained within 12 to 24 hr after the challenging dose was given. In the exception, the blood was tested several days after the challenging dose; it also revealed a high transaminase level.

A base-line study was not obtained in one patient receiving triacetyloleandomycin. This patient showed an elevated transaminase level after 21 days of therapy. She was then challenged, and high transaminase levels reappeared. The combined data from these studies are given in Tables 1 and 2 and in Figs. 1 and 2.

TABLE 1. *Patients developing evidence of hepatotoxicity*

| Drug and series | No. of patients | Evidence of hepatotoxicity | Involvement |
|---|---|---|---|
| | | | % |
| Total | 152 | 24 | 15.79 |
| Propionyl erythromycin ester lauryl sulfate (1st series) | 80 | 10 | 12.45 |
| Propionyl erythromycin ester lauryl sulfate (2nd series) | 13 | 5 | 38.46 |
| Triacetyloleandomycin (1st series) | 48 | 5 | 10.41 |
| Triacetyloleandomycin (2nd series) | 11 | 4 | 36.36 |

TABLE 2. *Adverse side effects appearing between 14 and 21 days of therapy*

| Drugs and series | No. of patients | Ict. Ind. | Serum Bil | Thym. turb. | Cephalin flocc. | SGPT | SGOT | Alkaline phosp. | Total eosinophil count | Periph. eos. count | Clinical symptoms | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | | | | | Without jaundice | With jaundice |
| Total | 152 | 12 | 5 | 5 | 6 | 24 | (7)* | 7 | (5)* | 15 | 9 | 2 |
| Propionyl erythromycin ester lauryl sulfate (2nd series) | 80 | 7 | 4 | 2 | 2 | 10 | Not done | 1 | Not done | 7 | 3 | 2 |
| Propionyl erythromycin ester lauryl sulfate (2nd series) | 13 | 2 | 0 | 0 | 0 | 5 | 2 | 2 | 4 | 4 | 3 | 0 |
| Triacetyloleandomycin (1st series) | 48 | 0 | 0 | 1 | 1 | 4 | Not done | 0 | Not done | 0 | 0 | 0 |
| Triacetyloleandomycin (2nd series) | 11 | 3 | 1 | 2 | 3 | 5 | 5 | 4 | 1 | 4 | 3 | 0 |
| Percent | | 7.82 | 3.28 | 3.28 | 3.94 | 15.78 | 29.12† | 4.6 | 20.0† | 9.87 | 5.91 | 1.32 |

*Figures are not complete since these tests were not done in first series.

†Percentage included only those in second series.

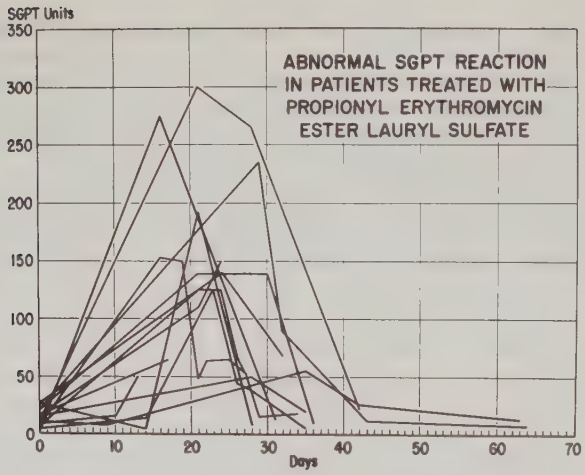


FIG. 1. *Abnormal SGPT reaction in patients treated with propionyl erythromycin ester lauryl sulfate.*

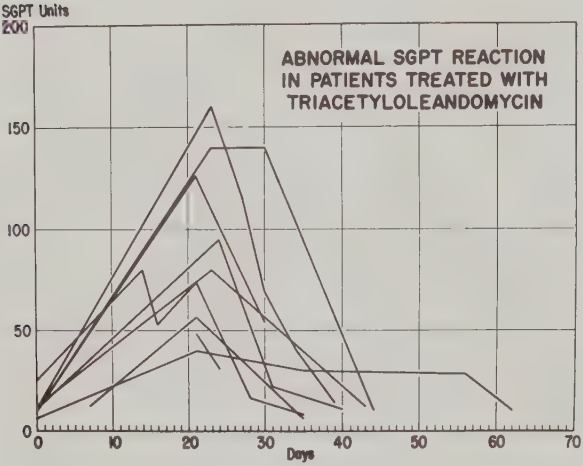


FIG. 2. *Abnormal SGPT reaction in patients treated with triacetyloleandomycin.*

I originally reported that in the group receiving triacetyloleandomycin no patient developed any clinical symptoms and no attempt was made to "challenge" those developing abnormal liver-function values. In the second series, three patients developed clinical symptoms without jaundice and three patients received challenging doses

after all signs and symptoms had reverted to normal. These three challenged patients responded with a repetition of the abnormal laboratory values and the clinical symptoms. It therefore, becomes evident that, where it was previously necessary to suppose a causal relationship of triacetyloleandomycin to the hepatic dysfunction, it is now evident that such a relationship truly exists.

One of the many factors which at the present time remains unexplainable is the peripheral eosinophilia and the eosinophilic infiltration of the hepatic tissue seen on biopsy. This eosinophilic infiltration has been verified by other investigators whose work is as yet unpublished.

## Discussion

The involvement of these two antibiotics as hepatotoxic agents, although important, is of considerably less significance than the overall picture of what is happening to the human organism as a result of these and other drugs. One must consider that here are two drugs which have, until recently, been considered relatively free of side effects. Both have been available for several years. Many millions of doses have been administered to millions of patients. The manufacturer of the erythromycin salt estimates that 15 million courses of treatment have been given. Only one isolated report, of three patients with jaundice due to triacetyloleandomycin in children who had been given the drug in doses of 100 mg per kg of body weight, exists.

Why has not the hepatoxic potential of these drugs been recognized? I offer these four reasons: (i) These drugs are less frequently used in excess of 14 days than they are in shorter courses of therapy. (ii) Jaundice or severe illness is relatively infrequent, occurring in only three patients in my original series of 80 cases on propionyl erythromycin ester lauryl sulfate and none in my series of cases on triacetyloleandomycin or the subsequent series of patients treated with both drugs. When jaundice does occur it may be diagnosed as infectious hepatitis. (iii) The clinical symptoms are minor in the largest percentage of patients developing hepatic dysfunction, and are frequently vague enough so that they are not associated with chemotoxicity. (iv) It is unusual that base-line laboratory studies are performed prior to administration of antibiotics and then repeated at regular intervals during therapy. Since demonstration of hepatotoxicity may be frequently solely on a laboratory level, it may be completely overlooked.

In recent months an extremely large number of cases of infectious hepatitis has been reported throughout the United States. For September 1961, 4,449 cases were reported, as contrasted to 3,139 for September 1960. For the first 9 months of 1961, the number of

cases reported was 57,275; this is an approximate increase of 100% above that for a comparable period of 1960 (U.S. Public Health Service Statistics).

Is it not possible that some of these cases reported as viral hepatitis are actually drug-induced rather than of viral etiology? When one examines the statistical reports of 1 to 5% mortality from infectious hepatitis (Davidson, 1955), one must consider the possibility that some of these are cases of chemohepatotoxicity.

In spite of the high rate of hepatic dysfunction in the series reported here, it is important to emphasize that I do not suggest that these drugs be proscribed. They are extremely valuable agents. I merely maintain that their hepatotoxic potential should be recognized, and that those patients receiving them should be watched more carefully than they have been in the past.

It must be emphasized that in both of these series, every case of hepatic dysfunction was reversible when the drug was discontinued. It is true that the hepatic abnormalities were promptly discovered. It is not known what would have happened if they had not been recognized and the drug continued.

Therefore, to reduce hepatic dysfunction to a minimum, liver profile and hemotologic studies should be obtained prior to giving the drug, if these drugs are to be administered in excess of 2 weeks. The liver profile should include: serum bilirubin, SGTP and SGOT, alkaline phosphatase, cephalin flocculation, and thymol turbidity. Thereafter, these tests should be repeated at short intervals. Other liver function studies, such as the Bromsulphalein and lactic dehydrogenase tests, should be included. However, the Bromsulphalein test is difficult to do on a routine basis in a private practice. Therefore, I have not included it in all cases. Steigmann (1930) suggested that testing be done at intervals of 7 days, but this would be extremely difficult in any but institutionalized patients.

Howard Ticktin, of George Washington University Medical School, has revealed to me that he has conducted a study on 50 patients treated with triacetyloleandomycin. He was able to verify my results; however, instead of the 10.41% abnormality which I originally reported, he detected hepatic dysfunction in 74% of his patients. Liver biopsies indicated a mixed type of hepatitis. Although cholestasis was present, evidence of hepatocellular damage was prominent.

### Acknowledgments

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## Literature Cited

Davidson, W. D. 1955. Viral hepatitis: risk from blood and plasma transfusion. J. Podiat. **46**: 717-723.

Koch, R., and L. D. Assay. 1958. Oleandomycin, a laboratory and clinical evaluation. J. Pediat. **53**:676-682.

Kohlstaedt, K. G. 1961. Propionyl erythromycin ester lauryl sulfate and jaundice. (Letter) J. Am. Med. Assoc. **178**: 89-90.

Robinson, M. M. 1961. Antibiotics increase incidence of hepatitis. (Letter) J. Am. Med. Assoc. **178**: 89.

Salm, R. 1953. Acute necrosis of liver following chloramphenicol therapy. Edinburgh Med. J. **60**: 334.

Steigmann, F. 1930. The early recognition of drug induced liver disease. Med. Clinics, North America **44**:183-192.

# EVALUATION OF A NEW SULFONAMIDE IN UROLOGICAL PRACTICE

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With modern chemotherapeutics, the acute, uncomplicated infections of the urinary tract are seldom difficult to cure (Kass, 1955). It is the chronic and recurrent infection that presents the greater problem to the physician.

Of these conditions, pyelonephritis is both the most frequently undiagnosed and the most difficult to manage (Kass, 1955). It is a more common entity than is suspected and is notably resistant to cure (McCabe, Jackson, and Grieble, 1959). Considering that pyelonephritis has been implicated in such disorders as hypertension, chronic renal insufficiency, toxemia of pregnancy, and stone formation (Beeson, 1955), the urologist cannot but regard its early diagnosis and vigorous treatment as imperative.

The magnitude of the problem becomes evident upon review of statistical studies showing that 6 to 20% of routine hospital autopsies revealed recently active, and old healed pyelonephritis; yet, in only one-fifth of these cases had clinical diagnosis of the disease been made (Jackson, Dallenbach, and Kipnis (1955).

The discrepancy between autopsy findings and antemortem diagnoses suggests a high incidence of inapparent pyelonephritis in the general population (Sanford, 1959). There is additional clinical evidence of the presence of continued active, occult renal infection: in prognosis studies of the pyelitis of infancy (usually considered a self-limited illness), long-term re-evaluation of 133 patients, followed for from 5 to 23 years, showed a frequency of 47% residual renal abnormalities. These included at least one of the following conditions: renal damage, persistent pyuria, or positive urine cultures; yet the majority of these patients were completely asymptomatic at the time of follow-up study (Wharton, Gray, and Guild, 1937; Woodruff and Everett, 1954; Macaulay and Sutton, 1957).

The clinical picture in chronic pyelonephritis is not characteristic, and therefore diagnosis is often difficult. The chronic case may show little evidence of active infection, the symptoms for which medical aid is sought are constitutional manifestations due to a slowly progressing renal insufficiency, rather than symptoms referable to the urinary tract (Lowsley and Kirwin, 1956).

Recurrent pyelonephritis may indicate either incomplete cure of the initial attack, or a new attack after healing. In either case, the import is serious, for each new attack causes further destruction of renal tissue (Lowsley and Kirwin, 1956).

The natural course of the disorder has been described as a series of separate episodes of infection, a disease continuum wherein the acute exacerbations are linked by an asymptomatic, but active infection that is constantly taking its toll of renal function (Jawetz, 1954).

If one accepts the "link" between episodes of the acute phase, or between the acute and end stage, pyelonephritis is indeed difficult to detect.

It has been suggested that "silent" or asymptomatic bacteriuria may be a manifestation (Sanford, 1959). MacDonald and co-workers (1957), found in 100 unselected autopsies that true bacteriuria (defined by them as greater than 100,000 bacteria per ml of urine) correlated well with the presence of pyelonephritis; they suggested that such a relationship may obtain during life. Kass (1957) noted that when bacteriuria was eliminated during pregnancy acute pyelonephritis did not occur, whereas 50% of untreated patients developed overt infection. Jackson, Grieble, and Knudsen (1958) estimate that 5% of patients with low degrees of bacteriuria actually have pyelonephritis.

It seems apparent, then, that bacteriological study alone may detect inapparent pyelonephritis, even though detection of the abacteriuric phase remains elusive.

The organisms most frequently identified in renal infections are the coli-aerogenes group, *Pseudomonas, Proteus, Staphylococcus aureus, S. albus,* and *Streptococcus faecalis,* against which, with the last being an exception, the sulfonamides are preponderantly effective (Lowsley and Kirwin, 1956).

Chronic infections of the upper tract, however, require extensive and adequate chemotherapy. Organisms imbedded in renal tissue, as well as any descending invasion of the lower tract, need prolonged contact with the antimicrobial agent. Since the duration of treatment affects the outcome of pyelonephritis, the drug chosen must be of low toxicity as well as of proven antibacterial activity against the infecting organism (Lowsley and Kirwin, 1956). The sulfonamides adequately fulfill both requirements.

Recently, a new sulfonamide, 5-methyl-3-sulfanilamido-isoxazole (Gantanol), was made available to us for clinical trial. Closely related to sulfisoxazole, preliminary clinical studies indicate that this new agent attains similarly high blood levels but sustains them longer, maintaining a therapeutic serum concentration for over 12 hr after a single 2-g dose (Brandman and Engelberg, 1960). In laboratory studies, its high solubility at the critical pH range (5.5 to 7.4) of urine was established, and Boger and Gavin (1960) found its excretion rate to be intermediate in terms of other long-acting sulfonamides.

Clinical trials with Gantanol have demonstrated its antibacterial efficacy against both gram-negative and gram-positive organisms (Kiser et al., 1961). In a double-blind study excellent results were obtained by Kiser and his group in 80% of acute, uncomplicated urinary tract infections, as well as in 45% of chronic cases with complicating obstructions. Side effects were reported to be minimal; only 1 patient of the 48 in the study experienced nausea and vomiting, attesting to the drug's relative freedom from toxicity.

On the basis of these favorable preliminary findings, it was decided to test this new sulfonamide in a variety of genitourinary infections, including ten cases of pyelonephritis.

## Materials and Methods

Gantanol was administered to 50 patients (30 males and 20 females), ranging in age from 18 to 78 years, the average age being 52 years.

The infections treated were cystitis, acute and chronic, 24 cases; pyelonephritis, 10; prostatitis, with and without cystitis; gonorrhea; urethritis; epididymitis; and cystitis with prostatic cancer, in order of decreasing frequency.

The average dosage schedule of Gantanol was 2 g initially, followed by 1 g three times a day, maintained for from 5 to 12 days. Five patients received other medication in conjunction with the test drug; four patients with penicillin-resistant gonorrhea were given tetracycline; one received a corticosteroid for acute epididymitis.

Urinalyses were done on 44 patients prior to and after treatment, with bacterial cultures obtained before and after therapy from 25 urines.

The criteria for evaluating results were as follows: "excellent," if bacteria-free urine cultures, together with complete remission of symptoms, were obtained, or, if complete remissions of symptoms and microscopically clear urine specimens (uncultured) resulted; "satisfactory," if the patients were asymptomatic after treatment, despite microscopic evidence of incomplete clearing of urine; "unsatisfactory," if neither symptoms nor urine showed significant change.

## Results

*Clinical response.* Of the 50 patients treated, 27 showed excellent results, becoming completely asymptomatic within 7 to 9 days; 10 showed satisfactory improvement, 12 unsatisfactory. Included in the unsatisfactory results were four cases of penicillin-resistant gonorrhea, treated with Gantanol for 3 days without response; the addition of tetracycline eradicated the gonococci.

Nine of the ten patients with acute and chronic pyelonephritis showed excellent response, i.e., complete remission of symptoms and bacteria-free urines in all those cultured. The tenth patient had no follow-up evaluation. Table 1 lists clinical response by disease entity.

TABLE 1. *Summary of results of treatment with Gantanol*

| Diagnosis | No. of patients | Excellent | Satis-factory | Unsatis-factory | No follow-up |
|---|---|---|---|---|---|
| Cystitis (chronic, 2) | 24 | 15(c-6)* | 4(c-3) | 5 | |
| Cystitis and prostatitis | 5 | 2(c-1) | 1(c-1) | 2(c-2) | |
| Cystitis | 1 | | | 1 | |
| Pyelonephritis | 10 | 9(c-5) | | | 1 |
| Prostatitis | 3 | | 3 | | |
| Epididymitis, acute | 1 | | 1 | | |
| Urethritis, nonspecific | 1 | | 1 | | |
| Urethritis, posterior | 1 | 1(c) | | | |
| Gonorrhea (1 with urethritis) | 4 | | | 4 T(c)† | |
| Total | 50 | 27 | 10 | 12 | 1* |

*Number of post-treatment cultures that were negative.

†Poor response until tetracycline added; post-treatment cultures negative.

Patient acceptance of the drug was good. No untoward side effects were experienced in this series.

*Antibacterial response.* Urine cultures became bacteriologically negative in 23 of 25 patients within the 5th to 7th day of treatment, but included in these results are the four gonococcal infections not eliminated until a tetracycline was employed adjunctively.

Nine types of organisms were identified; 21 of the 25 pathogens were gram-negative, the coli-aerogenes group being predominant. Table 2 lists the incidence of their occurrence and their eradication during treatment.

TABLE 2. *Evaluation of bacterial response to Gantanol*

| Organism | Before therapy | Eradicated by therapy | Persistent |
|---|---|---|---|
| Coli-aerogenes group | 15 | 13 | 2 |
| *Pseudomonas* | 1 | 1 | |
| *Conococcus* | 4 | 4-T* | |
| *Staphylococcus albus* | 1 | 1 | |
| *S. aureus,* hemolytic | 1 | 1 | |
| *Streptococcus faecalis* | 1 | 1 | |
| *Nonhemolytic staphylococcus* | 1 | 1 | |
| *Bacillus subtilis* | 1 | 1 | |
| Total | 25 | 23 | |

*Poor response with Gantanol for 3 days; tetracycline used adjunctively.

*Results of urinalyses.* Microscopic examination of centrifuged sediments was done on 44 urines before and after therapy. Gross pyuria (20 or more WBC/hpf) was present initially in 42. After treatment, 32 urines were clear, while 8 showed little or no change. Two with hematuria failed to clear.

Albuminuria (from 3 plus to 1 plus) present initially in nine patients was absent in six after treatment.

On the basis of this study, it appears that Gantanol would be of value, particularly in cases of acute and chronic pyelonephritis and cystitis. Its complete freedom from toxic manifestations, together with its antibacterial efficacy, commend it for continued trial for the eradication of genitourinary infections.

## Literature Cited


Beeson, P. B. 1955. Factors in the pathogenesis of pyelonephritis. Yale J. Biol. and Med. **28**: 81-104.

Boger, W. P., and J. J. Gavin. 1960. Sulfamethoxazole: comparison with sulfisoxazole and sulfaethiodole and cerebral spinal diffusion. Antibiotics & Chemotherapy **10**:572-580.

Brandman, O., and R. Engelberg. 1960. Metabolism studies with RO 4-2130, a new sulfonamide. Current Therap. Research **2**:364.

Jackson, G. G., F. D. Dallenbach, and G. P. Kipnis. 1955. Pyelonephritis: correlation of clinical and pathologic observations in antibiotic era. M. Clin. North America **39**:297-305.

Jawetz, E. 1954. Urinary tract infections. DM (Disease-a-Mo).

Kass, E. H. 1955. Management of infections of the urinary tract. Am. J. Med. **18**:764-781.

Kass, E. H. 1957. Bacteriuria and diagnosis of infections of urinary tract with observations on use of methionine as urinary antiseptic. A. M. A. Arch. Internal Med. **100**:709-714.

Kiser, W. S., P. Bormel, J. D. Young, and E. H. Silverstein. 1961. Clinical evaluation of RO 4-2130, a new sulfonamide. J. Urol. **85**:849-855.

Lowsley, O. S., and T. J. Kirwin. 1956. Clinical urology. 3rd ed. Williams & Wilkins Co., Baltimore. p.775.

Macaulay, D., and R. N. P. Sutton. 1957. Prognosis of urinary infections in childhood. Lancet **2**:1318-1320.

McCabe, W. R., G. G. Jackson, and H. G. Grieble. 1959. Treatment of chronic pyelonephritis. II. Short-term intravenous administration of single and multiple antibacterial agents. A. M. A. Arch. Internal Med. **104**:710-719.

MacDonald, R. A., H. Levitin, G. K. Mallory, and E. H. Kass. 1957. Relation between pyelonephritis and bacterial counts in urine, an autopsy study. New Engl. J. Med. **256**:915-922.

Sanford, J. P. 1959. Inapparent pyelonephritis - the missing link? J. Am. Med. Assoc. **169**:1711-1714.

Wharton, L. R., L. A. Gray, and H. G. Guild. 1937. Late effects of acute pyelitis in girls. J. Am. Med. Assoc. **109**:1597-1602.

Woodruff, J. D., and H. S. Everett. 1954. Prognosis in childhood urinary tract infections in girls. Am. J. Obstet. Gynecol. **68**:798-809.

# TREATMENT OF *STREPTOCOCCUS PYOGENES* INFECTIONS WITH A NEW SULFONAMIDE, 5-METHYL-3-SULFANILAMIDO-ISOXAZOLE

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One of the many important problems in military medicine is the control of acute upper respiratory infections. This is particularly true in large training areas, since newly arriving personnel constantly introduce fresh pathogens to the center. Changes of climate and daily regimen also must inevitably exert some influence on the resistance of the personnel to infections. In addition, the close proximity of personnel in such training areas will inevitably lead to rapid spread of infection.

Of the many upper respiratory infections encountered in such centers, a significant number is due to the $\beta$-hemolytic streptococcus, group A.

Lackland Air Force Base is one of the largest training centers for the U.S. Air Force in the continental United States, and past experience has indicated that there is an almost constant series of cases of pharyngitis, secondary to infections with $\beta$-hemolytic streptococcus, at all times, but that peak incidence tends to occur in the months of November and April.

In a study of 90 patients (Braden, Colmore, and Cummings, 1960), a new sulfonamide, 5-methyl-3-sulfanilamido-isoxazole (Gantanol) (Fig. 1), achieved a bacteriological conversion rate of 94%. The study was critically done, with post-therapeutic bacteriology, and also, in a number of cases, follow-up cultures over a period of several weeks. Only one relapse was reported to occur in this series, and it was pointed out in the paper that this was an extraordinary degree of activity which the new sulfonamide was exhibiting against the $\beta$-hemolytic streptococcus.

In reviewing the literature concerning the use of sulfonamides in the treatment of infection due to $\beta$-hemolytic streptococcus, it becomes apparent that sulfonamides have been rather ineffective in managing this particular bacterial invasion. In a series conducted by LaBoccetta et al. (1957), using 89 patients treated with sulfisoxazole to control the infections with $\beta$-hemolytic streptococcus, the conversion rate was only 13.5%.

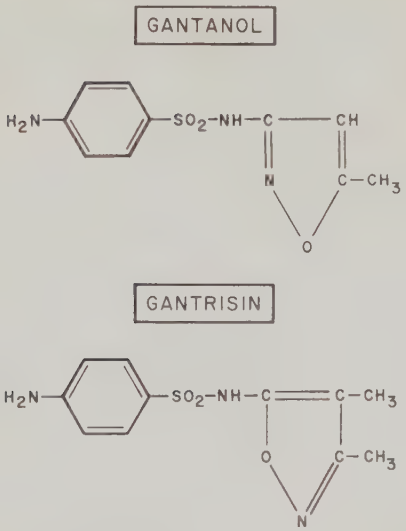


FIG. 1. *Structural formulas of Gantanol and Gantrisin.*

Weinstein, Madoff, and Samet (1960) made the following comments on streptococcal pharyngitis: "Sulfonamide compounds do not significantly alter the course of streptococcal pharyngitis. They do not eradicate the organisms from the throat, nor do they prevent the development of the late non-suppurative sequelae such as rheumatic fever and glomerulonephritis." Morris et al. (1956) reported the recovery of $\beta$-hemolytic streptococci 13 and 21 days after 5 days of therapy with full doses of sulfadiazine in 88 and 81% of cases, respectively. The same incidence of streptococcal recovery was observed in a comparable group of untreated patients. Penicillin therapy, on the other hand, eradicated the organisms from all but 1 or 2% of the subjects. Rheumatic fever was found to occur more frequently in patients given sulfonamides than in those to whom no drug was administered.

It will be quite apparent from the above isolated quotations that sulfonamides have been ineffectual in the management of this condition.

When a large number of inductees and military dependents at Lackland Air Force Base developed upper respiratory infections in which the pathogen was $\beta$-hemolytic *Streptococcus pyogenes*, the work of Braden et al. (1960) provided justification for a concentrated study of the action of Gantanol in this situation. It was recognized that it was of the utmost importance not only to inhibit, but to completely eliminate the *Streptococcus pyogenes* when it was encountered in this

group, because of the possible subsequent development of rheumatic fever or acute glomerulonephritis.

## Materials and Methods

The patient population of this study consisted of 138 males and 62 females, whose ages were distributed as follows: 6 to 15 years, 20; 16 to 25 years, 122; 26 to 35 years, 38; 36 to 45 years, 12; over 46 years, 3; and not recorded, 5.

All patients had upper respiratory infections exhibiting pretherapy cultures of $\beta$-hemolytic *S. pyogenes*. Throat cultures were repeated after the first week of therapy with Gantanol, and again 4 to 6 weeks after therapy. If the cultures were positive after the first week of treatment the patient was switched to penicillin medication.

The following uniform dosage schedule was maintained: 2 g stat-im, and 1 g every 12 hr. Duration of therapy was set at 2 weeks, unless the patient exhibited side effects or failed to respond to Gantanol. Blood levels with Gantanol, Gantrisin (Boger and Gavin, 1960), and Kynex (Boger, *unpublished data)* are compared in Fig. 2.

White blood counts, hematocrits, and urinalyses were done before and after therapy.

Sensitivity tests of the streptococci to 1, 5, 10, and 25 mg Gantanol discs were done in each case and reported as either clear zone of inhibition (+) or no inhibitory zone (-).

Pretherapy grouping of many strains was done by identifying the polysaccharide, and, in addition, subtyping of a random sample was done by identifying the M protein (Table 1).

TABLE 1. *Grouping and typing of pretherapy cultures of Streptococcus*

| $\beta$-Hemolytic streptococci | No. of cultures |
|---|---|
| Unclassified | 48 |
| Group A | 124 |
| Group A, type 22 | 22 |
| Group A, type 19 | 2 |
| Group A, type 17 | 2 |
| Group C | 1 |
| Group D | 1 |
| Total | 200 |

Results of therapy were rated as excellent if the cultures taken during and after therapy were both negative and if the patient was relieved of clinical symptoms without experiencing side effects from medication. A satisfactory rating was given patients whose response

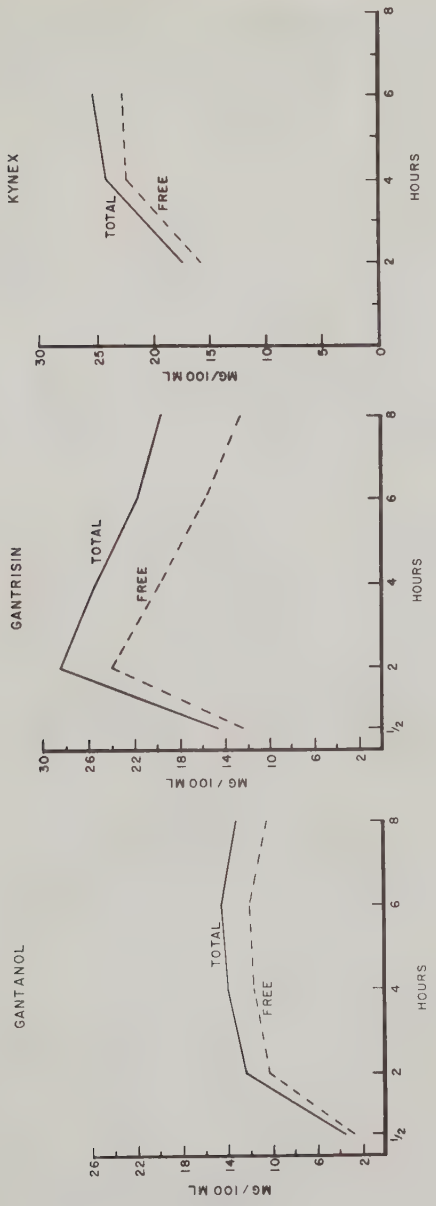


FIG. 2. *Plasma levels after a 2-g oral dose of Gantanol, Gantrisin, and Kynex.*

might be slower but whose infection was definitely cleared by the time the post-therapy culture was made. All patients with positive cultures after one week of medication were rated as showing an unsatisfactory response.

## Results

Response to Gantanol therapy was considered excellent in 137 cases (68.5%). These patients completed 14 days of therapy without experiencing side effects. Their throat cultures were negative after the first week of medication and at the second testing period from 4 to 6 weeks later.

Two cases were rated as satisfactory. One of these, a 10-year-old girl, had negative throat cultures during and after therapy, but on about the fourth treatment day she developed a mild skin rash and headache which persisted as long as she was on the drug (a 10-day period). The other case had a positive culture after 6 days of therapy, but a negative culture at the recheck period.

After one week of Gantanol therapy, 57 patients showed an unsatisfactory response and were then given penicillin.

Treatment was discontinued in four patients who experienced side effects after 1 or 2 days of medication. No "during-treatment" cultures were made in these four cases.

Regardless of the bacteriological response, the white blood count, in all but three or four patients, was reduced to a normal range; no significant changes in hematocrits were observed.

The urinalyses revealed no abnormalities attributable to medication.

In eight cases, including the four mentioned above, Gantanol was discontinued because of side effects (Table 2), which abated within 24 hr after withdrawal of the drug. In four cases the bacteriological response was not ascertained; of the four others, three had positive cultures and one had a negative culture. Most of these patients had more than one side reaction.

Although there is much evidence that a correlation between in vitro and in vivo action of the sulfonamides is erratic or perhaps nonexistent, sensitivity tests were made with Gantanol discs of 1, 5, 10, and 25 mg for each culture obtained. Early in the study some of the discs were placed by error on blood plates containing a fairly high concentration of para-aminobenzoic acid (PABA), which nullifies the bacteriostatic action of the sulfonamides. However, with Gantanol, the inhibition was found to be just as great if not greater than with PABA-deficient media. This seemed to indicate that Gantanol had a somewhat different mode of action from other sulfonamides, possibly interference with cell-wall synthesis, which might explain why it was effective against streptococci. The method worked quite well until

the latter part of the study, when it was found that none of the discs was inhibiting the growth of the streptococci, even in patients who were responding clinically and culturally.

TABLE 2. *Side effects resulting from Gantanol therapy**

| Effect | Incidence |
|---|---|
| Skin rash | 7 |
| Headache | 6 |
| Nausea and vomiting | 4 |
| Itching | 4 |
| Shivering sensations | 1 |

*Nine patients experienced side effects. Eight discontinued Gantanol after 1 to 3 days of treatment and side effects abated within 24 hr. In the one patient who continued on the drug, a mild skin rash and headache persisted throughout 10 days of therapy. Most patients had more than one side reaction.

A different agar, which had a higher concentration of PABA than the original, was being used at this time. It was decided, therefore, to transplant the streptococci, which had not responded to Gantanol discs in the original media, to Mueller-Hinton plates with 4 to 5% sheep blood added. When this was done, inhibition of the bacteria by the 10 and 25 mg discs was noted in most cases. A complete explanation of these findings is unclear. However, it appears that the concentration of PABA in the media is playing some role.

It should be remarked that the results of these tests were never equivocal; either there was a distinct clear zone of inhibition, or none at all (Table 3).

TABLE 3. *Correlation between cultures and disc sensitivity*

| Gantanol-treated cases after therapy | Disc size | Sensitivity zone | | Correlation |
|---|---|---|---|---|
| | | Present | Absent | |
| | *mg* | | | % |
| Negative cultures (138) | 1 | 14 | 124 | 10 |
| | 5 | 72 | 66 | 52 |
| | 10 | 95 | 43 | 69 |
| | 25 | 115 | 23 | 83 |
| Positive cultures (55) | 1 | 2 | 53 | 96 |
| | 5 | 9 | 46 | 84 |
| | 10 | 14 | 41 | 75 |
| | 25 | 34 | 21 | 38 |

The only conclusion to be drawn from these tests is that the disc-sensitivity method is inapplicable to the sulfonamides.

## Discussion

Although the work of Morris et al. (1956) and others indicates that sulfonamides are generally ineffective in eliminating streptococci in acute infections, in the present series Gantanol was effective in 71.5% of the cases. Follow-up cultures made from 4 to 6 weeks after termination of treatment with the drug continued to be negative in all who initially responded. There were no demonstrable relapses.

Although this conversion rate of 71.5% is not as high as that reported by Braden et al. (1960), nevertheless it is an unusual conversion rate for a sulfonamide against $\beta$-hemolytic streptococci, especially when one bears in mind that, in our present series, risks of reinfection are high.

There are two factors which may explain part of this discrepancy. Only two patients in the series of Braden et al. were dropped because of side effects, whereas we stopped Gantanol medication in eight at the first appearance of side effects, on the first or second day of medication. This action was taken because of the necessity of curing our patients as quickly as possible. No attempt was made to prolong Gantanol therapy if even minor complications appeared. The second factor was that patients were taken off the drug if the culture was positive after 5 to 8 days of medication, whereas five of the Braden patients, who were slow to respond within the 10-day treatment period, were continued on the drug for another week, by which time their cultures were negative.

This comparison indicates that perhaps some of our patients in whom the drug was discontinued after 5 to 8 days of therapy might have responded if therapy had been continued for a longer period (Table 4).

TABLE 4. *Results of treatment of streptococcal upper respiratory tract infections with employment of different agents at Lackland Air Force Base, Texas*

| Agent* | Follow-up cultures | Number positive | Conversion |
|---|---|---|---|
| | | | % |
| Penicillin (345) | 228 | 4 | 98.2 |
| Tetracycline (53) | 42 | 9 | 78.1 |
| Erythromycin (7) | 6 | 0 | 100 |
| Gantrisin (6) | 6 | 4 | 33.3 |
| Gantanol (200) | 200 | 57 | 71.5 |

*Numbers in parentheses indicate the actual number of patients treated.

Although Gantanol had a lower conversion rate than that of antibiotics used in streptococcal upper respiratory tract infections (98% in the case of penicillin when follow-up cultures were obtained in

in this hospital), its own surprising effectiveness indicates that more extensive use and investigation are in order.

TABLE 5. *Bacteriological conversion rates*

| Agent | Conversion |
|---|---|
| | % |
| Untreated controls | 5-48 |
| Sulfadiazine | 5-40 |
| Gantrisin | 22 |
| Tetracyclines | 55-76 |
| Erythromycin | 48-95 |
| Penicillin | 82-99 |

Comparison of the results of treatment at Lackland Air Force Base (Table 4) with results in the literature reveals a somewhat different conversion rate with the various agents (Table 5).

## Literature Cited

Boger, W. P., and J. J. Gavin. 1960. Sulfathiazole; comparison with sulfisoxazole and sulfaethidole and cerebrospinal fluid effusion. Antibiotics & Chemotherapy 10:572-580.

Braden, B., J. P. Colmore, and M. M. Cummings. 1961. Experience with a new sulfonamide in the treatment of streptococcal pharyngitis. Antimicrobial Agents Ann., p. 54-57.

LaBoccetta, A. C., M. Rosenberg, S. Harris, and T. N. Harria. 1957. Scarlet fever, results of a controlled study of 609 patients treated with penicillin and sulfisoxazole. Am. J. Med. 22: 458-468.

Morris, A. J., A. Chamovitz, F. J. Catanzaro, and C. H. Rammelkamp. 1956. Prevention of rheumatic fever by treatment of previous streptococcic infections. J. Am. Med. Assoc. 160: 114-117.

Weinstein, L., M. A. Madoff, and C. M. Samet. 1960. The sulfonamides. New Engl. J. Med. 263: 900-907.

# DIMETHOXYPHENYL PENICILLIN IN THE PREVENTION OF STAPHYLOCOCCAL COLONIZATION IN NEWBORN INFANTS

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Infection acquired during the newborn period represents one of the most common and frequently serious expressions of staphylococcal disease. Not only the ubiquitous distribution of staphylococci but also the rapid emergence of antibiotic-resistant patterns have served to accentuate the problem in newborn nurseries. It is well known that the newly born infant who is colonized by staphylococci during his stay in the newborn nursery may not exhibit clinical manifestations of disease until some time later. Thus, it is of great importance to determine if an infant has acquired staphylococci during this period and, if so, to study means of preventing it.

Dimethoxyphenyl penicillin (methicillin) appears to offer considerable promise in the treatment of staphylococcal infections. Methicillin is a synthetic penicillin whose most important characteristic is its property of being relatively resistant to the action of penicillinase. This quality should make it of value in the treatment of infections due to penicillin G-resistant strains of staphylococci. The present study was designed to elucidate the prophylactic effect of this drug in the prevention of staphylococcal colonization in newborn infants, particularly when used as continuing treatment during the nursery stay. It was anticipated that, concomitantly, further information would be obtained relating to the chronology and extent of staphylococcal colonization in the neonatal period.

## Materials and Methods

The studies were conducted over a period of approximately 5 months. Anterior nares cultures were obtained between 8:00 and 9:00 A.M., Monday and Tuesday mornings, on all infants admitted to the Newborn Nursery of the University Hospital during the previous 24-hour period. Cultures were repeated on the same infants at 36

to 100 hr of age. Infants were assigned randomly either to a "treatment" or "control" group on an alternate basis according to the time of delivery. Infants exhibiting abnormal, noninfectious symptomatology prior to the first culture were eliminated from the "control" and "treatment" groups and were included in a third group designated as "sick" infants. After anterior nares cultures were obtained, infants in the "treatment" group were given methicillin (25 mg/kg, daily) divided into two equal doses and administered every 12 hr intramuscularly. The drug was started within 4 hr after the first culture was obtained and continued until the second culture had been taken (average duration of therapy was 52.3 hr). Those in the "sick" group received no antibiotic treatment.

All anterior nares cultures were obtained by a research nurse assistant, who introduced sterile swabs to a depth of approximately 1.0 cm into both nostrils. The specimens then were inoculated immediately on brain heart infusion blood agar and "staphylococcus 110" media. After suitable incubation, staphylococcal isolates were categorized for hemolysis, pigmentation, and mannitol fermentation. Coagulase production was considered as negative if the staphylococci failed to coagulate plasma (diluted 1:3) within 3 hr in a 37 C water bath. Antibiotic susceptibility patterns were determined by the paper disc method. Bacteriophage typing was not done.

## Results

A total of 136 infants were studied; 58 infants were included in the "control" group, 50 in the "treatment" group, and 28 infants were placed in the "sick" group (Table 1). Initial cultures were obtained during the first 29 hr of life and repeat cultures were obtained between 36 and 100 hr of life. The duration between the first and second cultures averaged 54.5 hr in the "control" group, 52.3 hr in the "treatment" group, and 57.3 hr in the "sick" group of infants.

The great majority of initial cultures were negative for staphylococci. With respect to the presence of staphylococci, there was little difference between the initial cultures in the "control" and "treatment" groups. The second (repeat) cultures revealed 24 of 58 infants in the "control" group to harbor staphylococci, whereas the second cultures were positive for staphylococci in only 12 or 50 infants in the group treated with methicillin. The chi-square test for statistical significance of these differences in second cultures falls just at the borderline of significance at the 5% level.

Of the treated group, only nine of 45 infants whose initial cultures were negative harbored staphylococci on repeat cultures, whereas 22 of 55 controls exhibited staphylococcal implantation by repeat cultures. Of the three in the "control" group whose initial cultures were positive, one became negative at the time of the second culture,

TABLE 1. *Staphylococcal colonization in newborn infants*

| Infant group | Age (hr) | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | 0 to 5 | 6 to 11 | 12 to 17 | 18 to 23 | 24 to 29 | Total | 36 to 48 | 49 to 72 | 73 to 96 | 97 to 100 | Total |
| Control | | | | | | | | | | | |
| Negative for staphylococci | 19 | 12 | 12 | 11 | 1 | 55 | 2 | 16 | 16 | 0 | 34 |
| Positive for staphylococci | 1 (−)[a] | 1 (+)[b] | 0 | 1 (−)[c] | 0 | 3 | 3 (−) | 12 (−) | 8 (−)<br>1 (+) | 0 | 24 |
| Treated | | | | | | | | | | | |
| Negative for staphylococci | 11 | 11 | 9 | 12 | 2 | 45 | 3 | 23 | 11 | 1 | 38 |
| Positive for staphylococci | 0 | 0 | 2 (−)[d] | 2 (−)[e] | 1 (−)[f] | 5 | 0 | 7 (−) | 4 (−) | 1 (−) | 12 |
| Sick | | | | | | | | | | | |
| Negative for staphylococci | 7 | 4 | 5 | 8 | 0 | 24 | 0 | 5 | 1 | 0 | 6 |
| Positive for staphylococci | 0 | 1 | 1 | 1 (−)<br>1 (+) | 0 | 4 | 0 | 9 (−)<br>3 (+) | 9 (−)<br>3 (+) | 0 | 22 |

[a]Negative for staphylococci at 2nd culture (49 to 72 hr). In all cases, (+) = coagulase positive; (−) = coagulase negative.
[b]Changed to coagulase negative at 2nd culture (49 to 72 hr).
[c]Unchanged at 2nd culture (49 to 72 hr).
[d]1 negative at 2nd culture (49 to 72 hr), and 1 unchanged.
[e]1 negative at 2nd culture (49 to 72 hr), and 1 unchanged at 2nd culture (72 to 96 hr).
[f]Unchanged at 2nd culture (72 to 96 hr).

and two of five subjects in the treated group, whose initial cultures were positive, became negative at the time of reculture.

Although the group designated as "sick" infants was excluded from the comparative study, the initial and repeat cultures were interesting. Of 28 infants, only four were positive at the time of initial culture, but 22 were positive for staphylococcal implantation on repeat cultures.

Antibiotic susceptibility patterns revealed that, of 72 strains of staphylococci isolated from the study population, 39 were resistant to penicillin G, but only two were resistant to methicillin.

Methicillin administered intramuscularly in a daily dose of 25 mg/kg was well tolerated and there was no evidence of local or systemic untoward reactions.

## Discussion

Except under unusual circumstances, the normal infant may be expected to be born without indigenous staphylococci, but is highly likely to be colonized by these organisms by the end of the first week of life. Cunliffe (1949) found staphylococci in the anterior nares of only 8.6% of a series of subjects on the first day following birth, but the incidence had risen to nearly 100% by the end of the first week. Similarly, Rountree and Barbour (1950) observed that 89% of their study series of newborn infants were colonized at the end of the first week. These observations are in close agreement with those of Stoppelman (1954), who found that, although 80 to 85% of cultures taken during the first 12 hr after birth were sterile, staphylococci were present in 71% by the third day of life and in almost 100% at 10 days. Martyn (1949) has reported that 62% of a series of 130 newborn infants became colonized with staphylococci during the first 7 days after birth.

Two questions relating to such findings immediately become apparent. Does colonization by staphylococci significantly affect the health and well-being of newborns and, if so, what means of treatment are efficacious and applicable? Any attempts to evaluate the clinical significance of nasopharyngeal staphylococci should include consideration of the relative importance of the various strains of staphylococci usually involved, particularly with respect to coagulase production. It will be noted that, in the present study, the great majority of staphylococci cultured were "coagulase negative." However, coagulase production is not an absolute quality, but rather, one of comparative degree; and usual routine laboratory methods for the designation of staphylococci as "coagulase positive" or "coagulase negative" are quite arbitrary. Furthermore, it has been documented that coagulase-negative staphylococci can produce clinically important infections.

There is a paucity of information available regarding the association of illness in newborns with nasopharyngeal implantation of staphylococci. In the present study none of 136 infants had clinical staphylococcal infections during the nursery stay. Nevertheless, a considerably greater percentage of infants in the "sick" group became colonized at the end of 100 hr than was evidenced in the "control" or "treatment" groups. Since the abnormality of the "sick" infants could not be attributed to staphylococcal infection, it is suggested that the physiological conditions in these infants were more suitable for rapid colonization by staphylococci than in their more robust counterparts.

An earlier study on the effect of antibiotics on nasopharyngeal flora of premature infants revealed that oxytetracycline, combinations of penicillin and streptomycin, and of penicillin and sulfadiazine had little, or no, effect in suppressing the advent of staphylococcal implantation (Stoppelman, 1954). If anything, the incidence of staphylococci was greater in the treated group. Methicillin has been administered prophylactically in single doses to groups of newborns on the first day of life in an attempt to prevent staphylococcal implantation (Day et al., 1961). These authors reported a significant suppression of staphylococci for the first 3 days, but rapid colonization occurred by the end of the fourth day. The results of the present study suggest that a regimen of continuing methicillin therapy in the early neonatal period may be effective in reducing the incidence of nasal colonization with staphylococci.

To establish a basis for action in the event of an epidemic outbreak of staphylococcal infections in a newborn nursery, it is necessary to determine the effectiveness of various newer medications and procedures in the prevention of staphylococcal colonization. Our studies with methicillin are being continued in an effort to gain further information concerning the effectiveness of various dosages and regimens.

## Acknowledgment

The valuable assistance of Norma Divine, Hayden Mugler, and Sue Fuller in this study is acknowledged.

## Literature Cited

Cunliffe, A. C. 1949. Incidence of *Staphylococcus aureus* in the anterior nares of healthy children. Lancet 2:411-414.

Day, P. A., W. Osborne, W. Mesibov, H. Robidoux, and P. F. Wehrle. 1961. Dimethoxyphenyl penicillin: A study of its use as a prophylactic agent in the newborn nursery and in the treatment of infectious diseases in pediatric patients. *In* P. A. Bunn, (ed.), A symposium on the new dimethyoxyphenyl penicillin. State University of New York, Upstate Medical Center, Syracuse, p. 194-200.

Martyn, G. 1949. Staphylococci in the newborn. Brit. Med. J. 1:1146-1148.
Rountree, P. M., and R. G. H. Barbour. 1950. *Staphylococcus pyogenes* in new-born babies in a maternity hospital. Med. J. Australia 1:525-528.
Stoppleman, M. R. H. 1954. Effect of antibiotics on nasopharyngeal flora of premature infants. A. M. A. Am. J. Diseases Children 88:339-343.

# TREATMENT OF SERIOUS *PSEUDOMONAS* INFECTIONS WITH COLISTIMETHATE SODIUM

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Colistin is the generic name given to the antibiotic isolated from *Bacillus colistinus* by Koyama et al. (1950). It is a complex polypeptide related to polymyxin and has a molecular weight of about 969. It differs from polymyxin in that it does not contain the amino acid phenylalanine.

It has been demonstrated that colistin possesses marked in vitro bacteriostatic and bactericidal activity against many gram-negative organisms. Clinical studies to date have shown that it has been of value in the therapy of infections caused by *Pseudomonas aeruginosa*, an organism commonly resistant to other antibiotics. When administered intraumuscularly, colistin produces high serum levels lasting between 8 and 12 hr. Orally administered colistin (the sulfate of colistin) is only slightly absorbed. Unlike other polypeptide antibiotics, colistin, when administered in therapeutic doses, has not produced evidence of renal toxicity.

Many of the early studies were published in the foreign literature. The current cumulative bibliography (Warner-Lambert Research Institute, 1961) on colistin contains at least 175 titles. These studies showed favorable results in the treatment of enteric infections due to *Shigella* or pathogenic *Escherichia coli*. Gram-negative urinary tract infections responded favorably to colistin in 33 to 83% of the cases reported.

In a recent article, Hall (1960) carefully divided his cases into the following three categories: group A, major factors adverse to treatment such as obstructive uropathy or neurogenic bladder, repeated or indwelling catheter, chronic infection, severe underlying systemic disease, and undrained extensive abcesses; group B, minor factors adverse to treatment such as poorly controlled diabetes mellitus, prolonged corticosteroid therapy, and complicating local lesions; and group C, no factors adverse to treatment. There were 33 patients with factors seriously delaying recovery (group A). A number of these patients, however, were infected with organisms other

than *Pseudomonas*. Hall reported his results as follows: good, 40%; ineffective, 45%; and mixed, 15%. Good results were defined as complete eradication of infection or temporary suppression.

## Materials and Methods

The patients in this report are similar to those in Hall's group A. They were given colistin because they had *Pseudomonas* infections resistant to all other antibiotics, except polymyxin B. This was the only factor in selection. Evidence of in vitro sensitivity of the organism to colistin was available in every case before therapy with this drug was initiated.

Colistin was administered intramuscularly in a dose of 50 mg every 8 hr in all cases. In only one case was another antibiotic administered concurrently. All of the patients had been on other antimicrobial agents, including penicillin, tetracycline, and chloramphenicol, before colistin was started.

## Results

The *Pseudomonas* infections treated with colistin were as follows: chronic pyelonephritis, 4 cases; pulmonary infections, 8 cases (bronchogenic carcinoma, chronic bronchitis, and lung abcess, 3, 3, and 2 cases, respectively); and surgical wound infections, 2 cases.

Other conditions present in 14 colistin-treated cases were diabetes mellitus, uremia, hepatic cirrhosis, lymphoma, bronchogenic carcinoma, neurogenic bladder, carcinoma of the prostate, gastrointestinal bleeding, and severe emphysema. A number of the patients with malignancy were severely debilitated. Three patients had azotemia with blood urea nitrogen (BUN) values ranging from 40 to 140 mg per 100 ml.

*Bacteriological results.* Organisms were eradicated from the cultures in only 2 cases (14%). In one of these, an infection with *Aerobacter* sp. supervened. In two cases the organisms were temporarily suppressed. *Pseudomonas* reappeared, after therapy, in a lung abscess case and in one case of chronic pyelonephritis (at 2 and 4 weeks, respectively). In addition to the intramuscularly administered colistin, polymyxin B was instilled through the tracheotomy tube of the patient with lung abscess. In one case of abdominal wound infection, *Pseudomonas* became resistant to colistin after 6 days of therapy. There was a temporary overgrowth of *Proteus* sp. in one case of chronic pyelonephritis following cessation of colistin therapy (Table 1).

*Clinical results.* Of the 14 patients treated, six were clinically improved (43%); five showed no change (36%); and in three cases no

TABLE 1. *Bacteriological results of therapy with colistin*

| Type of infection | Number of patients | Eradication | Suppression | Avg length of therapy |
|---|---|---|---|---|
| | | | | *days* |
| Chronic pyelonephritis | 4 | 0 | 1 | 5.5 |
| Bronchogenic carcinoma | 3 | 0 | 0 | 4 |
| Chronic bronchitis | 3 | 1 | 0 | 8 |
| Lung abcess | 2 | 0 | 1 | 6 |
| Surgical wound | 2* | 1 | 0 | 6 |

*One case became resistant to colistin.

conclusions could be drawn (Table 2). The average duration of therapy was 6 days.

TABLE 2. *Clinical results of therapy with colistin*

| Type of infection | Number of patients | Number improved | Number unchanged |
|---|---|---|---|
| Chronic pyelonephritis | 4 | 0 | 2 |
| Bronchogenic carcinoma | 3 | 0 | 2 |
| Chronic bronchitis | 3 | 2 | 1 |
| Lung abcess | 2 | 2 | - |
| Surgical wound | 2 | 2 | - |

The only side effect occurred in one patient with uremia (BUN, 140 mg) and hepatic cirrhosis. This consisted of a generalized "burning" feeling after only 150 mg had been administered. There were no signs of renal toxicity, as measured by increase in BUN, or the appearance of albuminuria in any of the cases. Four patients died, all from underlying malignancies.

## Discussion

Therapy with colistin or polymyxin B was the only course available for managing the severe *Pseudomonas* infections of patients in this study. Colistin was chosen because of its lack of renal toxicity. Hall (1960) reported a good response (100%) in patients in his groups B and C. He felt that his results emphasized the importance of host factors in determining the outcome of treatment, a fact with which we agree. The final results in this series indicate that colistin was able to eradicate *P. aeruginosa* infections in only two (14%) of 14 patients who had severe underlying desease. Because the drug was available only for investigational purposes when the study was begun, the initial conservative dose of 150 mg per day was maintained in all cases for the purpose of comparison. For this reason, in addition

to the brief duration of therapy in several patients who died, it is possible that some of the patients received inadequate doses of the drug. Since so little toxicity was evident, higher doses might be used in treating serious *Pseudomonas* infections at the present time.

## Acknowledgment

Colistin was generously provided, as Coly-Mycin injectable (colistimethate sodium), by the Warner-Chilcott Laboratories, Morris Plains, N.J.

## Literature Cited

Hall, J. W. 1960. Colistin: analysis of a new antibiotic in 44 cases of systemic gram negative infections. Am. J. Med. Sci. 240:561-570.

Koyama, Y., A. Kurusawa, A. Tsuchiya, and K. Takauta. 1950. A new antibiotic, colistin, produced by spore-forming soil bacteria. J. Antibiotics (Japan) 3:457.

Warner-Lambert Research Institute. 1961. Colymicin bibliography. Morris Plains, N. J.

# *KLEBSIELLA* BACTEREMIA WITH REFERENCE TO COLISTIN THERAPY

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The occurrence of serious infection caused by gram-negative bacteria has been apparent in recent years. The infrequency of blood-stream invasion by gram-negative bacilli prior to the sulfonamide era and the increasing incidence since the introduction and widespread use of antibiotics has been well documented (Finland, Jones, and Barnes, 1959).

The mortality associated with *Klebsiella* bacteremia has been difficult to evaluate, but has been reported to be as high as 85% (Jervey and Hamburger, 1957). Undoubtedly such factors as the susceptibility of the age group over 50, the alcoholic, and the diabetic to *Klebsiella* infection are related to the aforementioned death rate (Austrian, 1959).

## Case Material

From January 1960 to August 1961, we observed 32 cases of *Klebsiella* bacteremia on the 225-bed University Medical Service at Kings County Hospital (Table 1).

TABLE 1. *Distribution of cases of Klebsiella bacteremia*

| Diagnosis | Total cases | Number survived | Number deaths | Percentage deaths |
|---|---|---|---|---|
| Pyelonephritis | 16 | 10 | 6 | 37.5 |
| Pneumonia | 12 | 7 | 5 | 41.5 |
| Biliary tract disease | 3 | 2 | 1 | 33.3 |
| Unknown | 1 | 1 | 0 | – |
| Total | 32 | 20 | 12 | 37.5 |

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## Results and Discussion

The mortality in this group, treated primarily with tetracycline or chloramphenicol plus streptomycin, was 37.5%, with the pneumonia cases exhibiting the poorest survival rate.

Sensitivity studies performed on 97 *Klebsiella* strains during this study revealed that 66% were resistant to sulfonamides, 28% resistant to tetracycline and streptomycin, and 20% resistant to chloramphenicol. All 97 were sensitive to kanamycin and only one strain was resistant to polymyxin B.

Studies in various laboratories in this country from 1951 to 1960 reveal that from 5 to 50% of *Klebsiella* strains are resistant to streptomycin, 12 to 40% resistant to broad-spectrum antibiotics, and 0 to 15% resistant to chloramphenicol (Lemke and Gates, 1951; Eisenberg, O'Loughlin, and Flippin, 1954; Warner-Chilcott Laboratories, Morris Plains, N.J., *unpublished data).* Higher degrees of resistance predominate in the most recent surveys. Although definite conclusions cannot be drawn from studies recorded in different laboratories over the past 10 years, it appears that we must be prepared to deal with an increased number of antibiotic-resistant *Klebsiella* infections.

Colistin, a polypeptide produced by *Bacillus colistinus,* has been highly effective in the treatment of infections due to gram-negative organisms (Carroll and Malette, 1961; Hall, 1960). After a single intramuscular injection, antibiotic activity is noted in the blood in 30 min and reaches a peak in 2 to 3 hr (McCabe, Jackson, and Konzij, 1959). Further observations indicate that the bacteriostatic and bactericidal concentrations for sensitive organisms are not appreciably different (Schwartz et al., 1959) and that the favorable response seen in urinary tract infections is due to the high concentration of the drug attained in the urine (Roberts and Kirby, 1960).

The bacteriostatic and bactericidal activity of colistin against *Klebsiella* has generally revealed a higher degree of sensitivity than streptomycin, tetracycline, or chloramphenicol (Petersdorf and Hook, 1960), and strains made resistant to streptomycin and the broad-spectrum antibiotics are not cross resistant to colistin (Schwartz et al., 1959).

Colistin-sensitivity studies of gram-negative bacteria recently isolated in our laboratory are listed in Table 2. A high degree of susceptibility can be noted to strains of *Klebsiella, Aerobacter, Escherichia, Paracolobactrum,* and *Alcaligenes faecalis.* The relative resistance of *Proteus* strains is in agreement with the findings in most laboratories (Petersdorf and Hook, 1960; Meleney and Prout, 1961).

After a review of the material just presented, it seemed advisable to prescribe colistin in cases diagnosed as *Klebsiella* bacteremia in an attempt to improve the rate of recovery.

TABLE 2. *Sensitivity studies of colistin*

| Organism | Number sensitive | Number moderately sensitive | Number resistant |
|---|---|---|---|
| *Klebsiella* | 7 | 2 | 1 |
| *Aerobacter* | 7 | 0 | 0 |
| *Escherichia coli* | 7 | 1 | 0 |
| *Proteus* | 2 | 0 | 6 |
| *Paracolobactrum* | 2 | 0 | 1 |
| *Alcaligenes fecalis* | 2 | 0 | 0 |

Colistin therapy was initiated in four cases of pneumonia and five cases of pyelonephritis (Table 3). In six patients it was the only antibiotic administered and in three patients colistin was combined with tetracycline. Steroids were specifically excluded in this study.

TABLE 3. *Manner of colistin therapy*

| Infection | Number of cases | Antibiotic used |
|---|---|---|
| Pneumonia | 2 | Colistin |
| Pneumonia | 2 | Colistin + tetracycline |
| Pyelonephritis | 4 | Colistin |
| Pyelonephritis | 1 | Colistin + tetracycline |

*Klebsiella* pneumonia with bacteremia occurred in four males with an age range of 38 to 64 years. Three patients were chronic alcoholics and one was a diabetic. Within the past 3 years, two of the four had been hospitalized for "pneumonia." Review of their records noted, in each instance, a prior pneumococcal pneumonia which responded to penicillin therapy. The patients presented an acute febrile illness, with respiratory symptoms of cough, sputum, dyspnea, and chest pain quite evident. Physical examination and chest X rays confirmed the diagnosis of an upper-lobe pneumonic process in one case and bilateral pneumonia in the remaining three. The white blood count revealed a leukocytosis of 15,000 or more in all four patients. A sputum smear was examined shortly after admission and gram-negative rods characteristic of *Klebsiella* were identified in each case. Initial antibiotic therapy for a presumptive *Klebsiella* pneumonia was thereby instituted within 2 hr of hospitalization. The importance of this initial sputum-smear examination cannot be overemphasized. It is well known that as much as a 12-hr delay in treatment of *Klebsiella* infection will markedly impair the possibility of a therapeutic success, no matter what antibiotic regimen is employed (Sale, Smith, and Wood, 1947). Subsequent cultures of the sputum and blood confirmed the presence of *Klebsiella pneumoniae*.

In contrast to the pneumonia series, four females and one male were treated for *Klebsiella* pyelonephritis and bacteremia. All five patients were over 60 years old and two were diabetic. Four of the five had received antimicrobial therapy within the past year for "kidney infections." In three cases sensitivity studies revealed resistance to at least one of the following drugs: tetracycline, chloramphenicol, and streptomycin. In two cases, resistance to all three drugs was encountered.

These five patients were treated within 24 hr after hospitalization with colistin alone or in combination with tetracycline.

Colistin dosage ranged from 2.5 to 5 mg per kg per day (or 150 to 300 mg), intramuscularly, in three or four divided doses. Therapy was continued for 1 to 2 weeks in seven of the nine cases and a clinical response was evident in the majority of cases within the first 3 days. All nine patients survived. However, our 10th case, treated since this paper was submitted, became our first fatality. This was a 79-year-old male treated with colistin for a *Klebsiella* pyonephrosis and bacteremia. Although colistin cleared his blood of *Klebsiella,* he succumbed with a *Proteus* superinfection.

Colistin toxicity was limited to pain at the injection site in one patient and vertigo in another. Nephrotoxicity was not observed in the pneumonia series. In three of the five cases with renal infection and bacteremia, the pretreatment blood urea nitrogen was elevated, and all returned to normal limits during therapy.

It is felt that the simultaneous administration of two antibiotics is the wisest course in *Klebsiella* infections, since strains of *Klebsiella* do not manifest uniform susceptibility to chemotherapeutic and antibiotic agents, and the emergence of bacterial resistance has been demonstrated (Austrian, 1959).

## Literature Cited

Austrian, R. 1959. Friedlander's pneumonia, p. 77-82c. *In* Practice of medicine, vol. 3. W. F. Prior Co., Inc., Hagerstown, Md.

Carroll, G., and W. F. Malette. 1961. The use of colymycin in chronic infections due to *Pseudomonas aeruginosa.* J. Urol. **85**:86-88.

Eisenberg, G. M., J. M. O'Loughlin, and H. F. Flippin. 1954. Distribution and in vitro antibiotic susceptibility of *Klebsiella.* J. Lab. Clin. Med. **43**:707-712.

Finland, M., W. F. Jones, Jr., and M. W. Barnes. 1959. Occurrence of serious bacterial infections since introduction of antibacterial agents. J. Am. Med. Assoc. **170**:2188-2197.

Hall, J. W. 1960. Colistin: an analysis of a new antibiotic in 44 cases of systemic gram-negative bacterial infection. Am. J. Med. Sci. **240**:561-570.

Jervey, L. P., Jr., and M. Hamburger. 1957. The treatment of acute Friedlander's bacillus pneumonia. A. M. A. Arch. Internal Med. **99**:1-7.

Lemke, C. E., and L. E. Gates. 1951. Distribution and antibiotic sensitivity in fifty-five strains of *Klebsiella pneumoniae.* J. Lab. Clin. Med. **38**:889-892.

McCabe, W. R., G. G. Jackson, and V. M. Konzij. 1960. Clinical and laboratory observations on the use of colistin in infections by gram-negative bacilli. Antibiotics Ann. 1959-1960, p. 80-88.

Meleny, F. L., and G. R. Prout, Jr. 1961. Some laboratory and clinical observations on Coly-Mycin (colistin) with particular reference to *Pseudomonas* infections. Surg. Gynecol. Obstet. 112:211-217.

Petersdorf, R. G., and E. W. Hook. 1960. The use of colistin in the treatment of infections of the urinary tract. Bull. Johns Hopkins Hosp. 107:133-142.

Roberts, C. E., Jr., and W. M. Kirby. 1961. Colistin in the treatment of hospitalized patients with *Pseudomonas* infections. Antimicrobial Agents Ann. 1960, p. 409-423.

Sale, L., Jr., M. R. Smith, and W. B. Wood, Jr. 1947. Studies on mechanism of recovery in pneumonia due to Friedlander's bacillus; effect of sulfonamide chemotherapy upon pulmonary lesion of experimental Friedlander's bacillus pneumonia. J. Exptl. Med. 86:249-256.

Schwartz, B. S., M. R. Warren, F. A. Barkley, and L. Landis. 1960. Microbiological and pharmacological studies of colistin sulfate and sodium colistinmethanesulfonate. Antibiotics Ann. 1959-1960, p. 41-60.

# ABSORPTION, EXCRETION, AND DISTRIBUTION OF COLISTIN

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Colistin is an antibiotic produced by *Bacillus polymyxa* var. *colistinus* (Koyamo et al., 1950). It is particularly active against *Salmonella* and *Shigella* organisms, as well as *Hemophilis pertussis*, *Escherichia coli*, and some strains of *Pseudomonas aeruginosa* and *Proteus vulgaris* (Nagai, 1951; Schwartz et al., 1960; Wright and Welch, 1960). It is commercially available in two forms, colistin sulfate and sodium colistinmethanesulfonate. Although sodium colistinmethanesulfonate is slightly less active than colistin sulfate, it is also less toxic (Schwartz et al., 1960; Wright and Welch, 1960) and is the drug of choice for parenteral medication. The clinical efficacy of this antibiotic has been the subject of a large number of papers in the literature, but certain parameters of its pharmacology have not been reported and are the subject of this report.

## Materials and Methods

The colistin used in this study was from a single lot (GMC 1001) of commercial material manufactured by the Kayaku Antibiotics Research Company, Ltd., Tokyo, Japan. (This material was generously supplied by Warner-Lambert Research Institute, Morris Plains, N.J. Colistin is marketed under the trademark Colymycin.) It was packaged in a 10-ml vial as a dry powder containing 1,000,000 units (30.8 $\mu$g) of sodium colistinmethanesulfonate and 2 mg of dibucaine hydrochloride. This powder was reconstituted with sterile distilled water immediately prior to administration. The claimed potency for this lot was validated by taking a random sample of nine vials and assaying each vial individually (Table 1).

The medication was administered to all patients by intramuscular injection, either into the deltoid muscle or the upper outer quadrant of the buttock. Venous blood specimens were drawn into heparinized syringes, promptly centrifuged, and the plasma harvested and placed in 10-ml vials which were stored at -4 C until assayed. Cerebrospinal fluid specimens, obtained at the time of diagnostic lumbar puncture, were stored in the same manner.

TABLE 1. *Individual potency of nine vials of colistin**

| Vial | Assayed potency* |
|---|---|
| | *units* |
| 1 | 1,150,000 |
| 2 | 875,000 |
| 3 | 1,075,000 |
| 4 | 965,000 |
| 5 | 965,000 |
| 6 | 1,075,000 |
| 7 | 1,250,000 |
| 8 | 1,000,000 |
| 9 | 1,325,000 |
| Average | 1,075,555 |

*Range (as per cent of claim), 87.5 to 132.5.

All assays were performed using an agar plate diffusion method which employs *Bordetella bronchiseptica* ATCC 4617 as the test organism. The method is a modification of the U.S. Food and Drug Administration method for the assay of polymyxin. As a control measure, all specimens from each part of this study were assayed during the same test.

It is essential to point out that the final dilution of the specimen and standard contains 10% phosphate buffer (pH 6.0). The use of this buffer increases the sensitivity of the assay and eliminates the effect of interfering substances. Readable inhibition zones are obtained with concentrations as low as 2.5 units per ml of standard, but it was found that 10% phosphate buffer, *per se*, gives diffuse, faint zones of inhibition, thus throwing into question the reliability of assays at this low level.

Although this effect is of little concern when high potency materials or pure standards are assayed, it is our opinion that assays indicating concentrations of less than 10 units per ml involve a large inherent error of assay, and absolute values cannot be too rigidly interpreted. All values below 10 units (0.33 $\mu$g) per ml reported in this study are subject to this qualification.

## Results

*Serum concentration and urinary excretion.* Six healthy adult volunteers were selected for the first part of this study. Each received an intramuscular injection of 1,000,000 units of colistin, and venous blood specimens were drawn immediately prior to the injection and at 2, 4, 6, and 8 hr thereafter. Fractional urine collections were made (0 to 2, 2 to 4, and 4 to 8 hr after medication). The

results (Table 2) are in general agreement with those reported by others (Schwartz et al., 1960; Wright and Welch, 1960).

TABLE 2. *Serum concentration and urinary recovery of colistin after a single intramuscular 1,000,000-unit dose*

| Pt. | Sex | Age | Wt | Serum concn (units/ml) | | | | | Urinary recovery (units) | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | 0 | 2 | 4 | 6 | 8 | 0-2 | 2-4 | 4-8 | Total |
| RL | F | 27 | 125 | <2.5 | 36.0 | 16.0 | 9.4 | <2.5 | 245,000 | 144,000 | 290,820 | 679,820 |
| HFr | F | 47 | 110 | <2.5 | 60.0 | 30.0 | 11.2 | 8.4 | 305,000 | 230,000 | 249,600 | 784,600 |
| HFi | F | 42 | 135 | <2.5 | 44.0 | 17.0 | 10.4 | <2.5 | 184,300 | 126,000 | 113,100 | 423,400 |
| JW | M | 40 | 168 | <2.5 | 36.0 | 14.4 | 6.0 | <2.5 | 39,936 | 560,000 | 400,000 | 999,936 |
| HC | M | 49 | 120 | <2.5 | 42.0 | 21.0 | 7.9 | 6.5 | 39,128 | 326,000 | 32,386 | 397,514 |
| PH | F | 18 | 94 | <2.5 | 37.0 | 18.4 | 5.4 | 5.4 | 211,120 | 240,000 | 41,730 | 492,850 |
| Average | | | | <2.5 | 43.0 | 19.5 | 8.4 | 3.4 | 170.747 | 271,000 | 187,939 | 629,686 |

In addition, seven elderly patients with urinary infections attributed to *E. coli* were treated with 1,000,000 units (30.8 μg) of

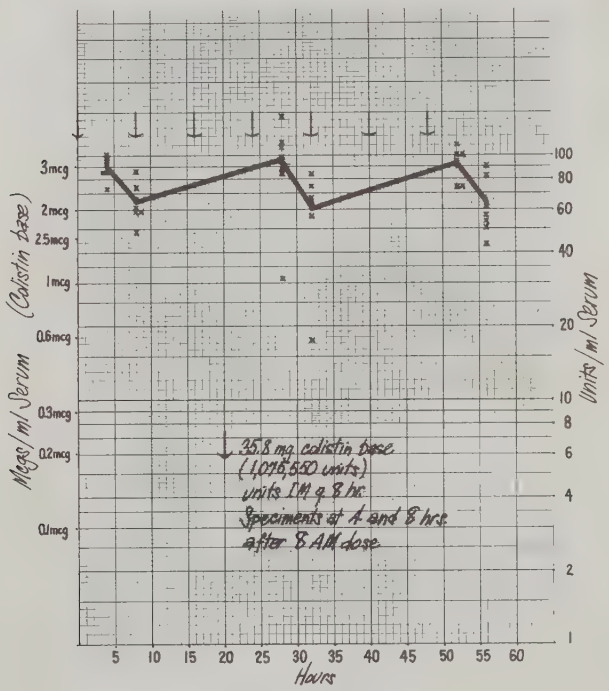


FIG. 1. *Serum concentrations of colistin during multiple dose therapy (seven patients; average age, 73).*

coliston three times a day (8 AM, 4 PM, 12 midnight). Blood specimens were obtained at 4 and 8 hr after the 8 AM dose for the first 3 days of treatment. This was done to determine whether there was any accumulation of the drug when a patient was placed on multiple dose therapy (Fig. 1).

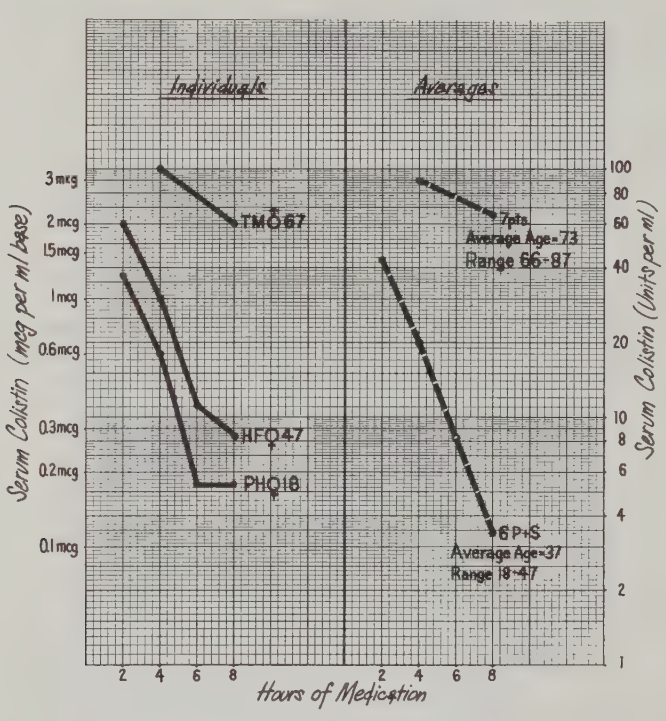


FIG. 2. *Influence of age on serum concentration of colistin after a single intramuscular injection of 35.8 mg (1,075,550 units) of colistin base.*

Although it is apparent that no accumulation of the drug occurred, as evidenced by peak concentrations of the same magnitude after multiple injections followed by similar rates of decline, it is interesting to note that the colistin serum concentrations are almost 5 times as high at 4 hr and 30 times as high at 8 hr, after initial treatment, in these elderly patients than in the younger volunteers (Fig. 2). A particularly striking difference is seen between volunteer P. H., age 18, and patient T. M., age 67. This suggests that aging compromises renal function in some patients in such a manner as to be reflected in higher serum concentrations with a slower rate of decline than is observed in persons with normal and "younger"

kidneys. It is our impression that the general statement made by Wright and Welch (1960) that "the serum concentrations diminished relatively rapidly and predictably" should be qualified. Our results show clearly that the excretion pattern for this antibiotic may be modified by the renal status of the patient, and that with age renal function tends to become impaired. The likelihood of toxicity in patients with impaired kidney function should be considered.

Because of a long-continuing interest in the absorption and excretion of antibacterial drugs, a study was designed to discover whether probenecid had any effect on the renal excretion of this compound. In a crossover pattern of study, five volunteers were studied on two different occasions. On the first day, three patients received 2 g of oral probenecid, 2 hr prior to an intramuscular injection of 500,000 units (16.66 $\mu$g) of colistin. The other two patients received only the intramuscular injection of colistin. With the reversal of medication, the same study was carried out 5 days later.

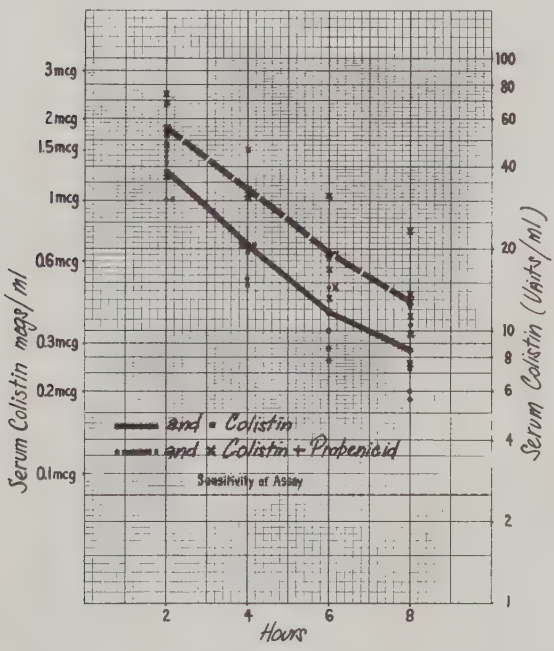


FIG. 3. *Effect of probenecid (2 g, orally) on the serum concentration of colistin (16.66 mg base, intramuscular).*

This lapse of time between phases of the study was dictated by the necessity of having probenecid cleared from the circulation. There is a small but definite increase in the serum colistin concentration after ingestion of probenecid (Fig. 3), suggesting that coliston may be excreted by the renal tubules of man.

At all points of observation, the findings are almost twofold higher after probenecid. Further, the values assayed are in the range of the best performance of the assay procedure. These data, together with the urinary recoveries of 40 to 80% of the administered dose (Table 2), suggest that there may be a tubular component involved in the excretion of colistin.

*Cerebrospinal fluid diffusion.* A total of 55 patients, all of whom were scheduled for a diagnostic lumbar puncture, was treated at various times (6, 8, and 12 hr) with a single intramuscular dose of

TABLE 3. *Colistin concentrations (units/ml)* in paired specimens of plasma and cerebrospinal fluid (CSF) after single intramuscular 1,000,000-unit dose*

| Hours after dose | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 6 | | | | | 8 | | | | | 12 | | | | |
| Pt. | Age | Sex | Plasma | CSF | Pt. | Age | Sex | Plasma | CSF | Pt. | Age | Sex | Plasma | CSF |
| AT | 15 | F | 9.6 | 10.0 | BK | 69 | F | 6.2 | 9.2 | HG | 45 | F | 5.2 | 8.3 |
| MN | 50 | F | 12.9 | 10.5 | AT | 15 | F | 11.9 | – | RR | 60 | F | 6.0 | 8.8 |
| SJ | 30 | F | 12.9 | 11.0 | MG | 41 | F | 7.4 | 4.6 | ME | 14 | M | 4.9 | 7.4 |
| EG | 23 | M | 11.3 | 8.8 | LC | 43 | F | 7.8 | 4.6 | MW | 50 | M | 2.3 | 10.0 |
| DW | 30 | M | 15.3 | 7.8 | LB | 63 | F | 9.3 | 8.8 | JG | 32 | M | 2.7 | 4.9 |
| NP | 33 | M | 16.0 | 5.5 | CS | 30 | F | 9.6 | 9.2 | EL | 42 | M | 3.8 | 4.9 |
| GM | 14 | M | 8.9 | 8.3 | AH | 26 | F | 16.4 | 7.4 | FH | 38 | M | 5.8 | 9.2 |
| JM | 40 | M | 9.2 | 5.8 | NM | 27 | M | 6.9 | 5.5 | BW | 38 | M | 4.9 | 5.5 |
| SD | 50 | M | 12.5 | 7.4 | GM | 14 | M | 10.2 | N.S. | DP | 49 | F | 5.6 | 8.3 |
| DB | 44 | M | 8.9 | 7.8 | FG | 46 | M | 10.9 | 5.2 | FA | 34 | F | 6.6 | 8.8 |
| AW | 31 | M | 4.9 | 7.8 | RB | 25 | M | 7.2 | 4.2 | GU | 46 | F | 4.3 | 4.4 |
| HJ | 14 | M | 10.9 | 6.6 | RN | 74 | M | 9.9 | – | FU | 24 | F | 8.4 | 7.0 |
| JW | 31 | M | 8.9 | 10.0 | GV | 41 | M | 5.6 | 10.0 | JK | 16 | M | 5.8 | 6.2 |
| | | | | | JB | 33 | M | 7.6 | 6.2 | DP | 23 | M | 2.5 | 7.4 |
| Average | | | 10.9 | 8.2 | RS | 36 | M | 6.8 | 8.3 | HC | 39 | M | 2.8 | 8.8 |
| | | | | | PM | 35 | M | 11.3 | 9.2 | CH | 66 | M | 3.7 | 7.0 |
| | | | | | | | | | | JS | 25 | M | 2.5 | 5.2 |
| | | | | | Average | | | 9.1 | 7.1 | CH | 60 | M | 5.8 | 8.3 |
| | | | | | | | | | | JB | 15 | M | 8.1 | 8.8 |
| | | | | | | | | | | AP | 39 | M | 2.5 | 7.0 |
| | | | | | | | | | | WB | 25 | M | 2.9 | 5.8 |
| | | | | | | | | | | LN | 24 | M | 1.7 | 8.3 |
| | | | | | | | | | | AH | 14 | M | 2.4 | 6.2 |
| | | | | | | | | | | GL | 23 | M | 2.8 | 6.2 |
| | | | | | | | | | | WP | 23 | M | 3.2 | 8.8 |
| | | | | | | | | | | RK | 28 | M | 5.2 | 7.8 |
| | | | | | | | | | | Average | | | 4.3 | 7.2 |

*30.8 units = 1 μg.

1,000,000 units (30.8 $\mu$g) of colistin. Table 3 presents the colistin determinations on simultaneous specimens of plasma and cerebrospinal fluid. It is of interest that all of the patients showed antibiotic activity in the spinal fluid, indicating that there is a sufficient concentration of unbound drug to diffuse promptly through the uninflamed meninges of man. This evidence of diffusion into the cerebrospinal fluid has direct therapeutic implications in connection with the treatment of those uncommon but troublesome cases of meningitis caused by *P. pyocyaneus*. Although quantitative values have been reported (Table 3) for cerebrospinal fluid diffusion, the values should be interpreted with some reservation, since 10% phosphate buffer will *per se* cause *some* inhibition of the test organism. The values reported imply that some diffusion occurs.

## Acknowledgments

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## Literature Cited

Koyamo, Y., A. Karosasa, A. Tsuchiya, and K. Takauta. 1950. A new antibiotic "colistin" produced by spore forming soil bacteria. J. Antibiotics (Japan) 3A:457-458.

Nagi, K. 1951. Studies of colistin, a new antibiotic against gram-negative bacilli. J. Antibiotics (Japan) 4A:565-567.

Schwartz, B. X., W. R. Warren, F. A. Barkley, and L. Landis. 1960. Microbiological and pharmacological studies of colistin sulfate and sodium colistinmethanesulfonate. Antibiotics Ann. 1959-1960, p. 41-60.

Wright, W. W., and H. Welch. 1960. Chemical, biological, and clinical observations on colistin. Antibiotics Ann. 1959-1960, p. 61-74.

# X-5079C, A NEW POLYPEPTIDE ANTIFUNGAL ANTIBIOTIC ACTIVE AGAINST SYSTEMIC MYCOSES

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In the course of our search for new antibiotics, a hitherto unidentified *Streptomyces* species, X-5079, was isolated from a soil sample from the grounds of Roche Park, Nutley, N.J. This culture, in submerged shake-flask growth, produced a mixture of antibacterial and antifungal antibiotics. When a crude concentrate showing good in vitro activity against the mold *Paecilomyces varioti* was found to be active systemically against histoplasmosis in mice, isolation of the pure substance was undertaken.

A number of recent publications have discussed assay methods (Grunberg, Berger, and Titsworth, 1961; Emmons and Piggott, 1961) and the results of chemotherapeutic studies with this antibiotic in animals (Grunberg et al., 1961; Emmons, 1961) and in man (Utz, Andriole, and Emmons, 1961; Emmons and Piggott, 1961; Andriole, Utz, and Sabesin, 1961; Louria, 1961). This paper will therefore devote itself primarily to the microbiological production, chemical isolation, and the physical and chemical properties of the new antibiotic.

## Materials and Methods

A detailed description of the organism which produces the antibiotic, *Streptomyces* sp. n. X-5079, will be given in a subsequent publication. (A culture of *Streptomyces* sp. n. X-5079 has been deposited in the collection of microorganisms of the U.S. Department of Agriculture, Northern Utilization Research and Development Division, Peoria, Ill., under the registration number NRRL 2831.) The culture is aerobic, mesophilic, and characterized by growth in the form of a much-branched mycelium, with a typical aerial mycelium, generally white, then turning to light gray and dark gray. The vegetative mycelium forms conidia, which are borne in chains in aerial

hyphae with much spiral formation. In the laboratory, *Streptomyces* X-5079 was grown in 500-ml Erlenmeyer flasks containing 100 ml of medium, adjusted to pH 6.5 to 7 before sterilization, and incubated at 28 C on rotary or reciprocal shaking machines. In the pilot plant, 50 to 4,000 gallon fermentation kettles were employed, fitted out for aseptic operation with aeration, agitation, temperature, and antifoam controls. A cup-plate agar diffusion method was used for assay, with the test mold *Paecilomyces varioti* (Grunberg et al., 1961).

## Results

*Fermentation.* In shaken-flask culture, *Streptomyces* X-5079 grows well on many nitrogen sources, such as soy bean flour, cottonseed meal, fish meal, peptones, yeast, distiller's dried solubles, and casein hydrolyzates. On synthetic media, the single amino acids alanine, arginine, glutamic acid, lysine, and valine support good growth. Many carbon sources may be utilized for growth, such as glucose, sucrose, starch, lactose, maltose, and mannitol. The best medium for antibiotic production, however, contains 2% soy bean flour, 2% brown sugar, 0.5% cornsteep liquor concentrate, 0.1% $K_2HPO_4$, and lard oil as an antifoam agent. Representative antibiotic yields in shake flasks are presented in Table 1. Assays were carried out on whole-broth samples, diluted with 0.67 M phosphate buffer (pH 6.2) so as to produce inhibition zones within the range of reliable calculation (diameters of 16 to 23 mm).

TABLE 1. *Effect of various nitrogen sources on the production of antibiotic X-5079C in shaken flasks*

| Addition to basal medium* | *P. varioti* units/ml, whole broth, 6 days |
|---|---|
| Soy flour (Soyalose no. 103, Glidden), 2% | 52 |
| Protein peanut oil meal, 1%, distiller's dried solubles, 1%, meat meal digest (Armour), 1% | 46 |
| Dried corn fermentation solubles with grain (soluferm, Commercial Solvents Corp.), 2% | 28 |
| Menhaden fish meal, 2% | 29 |
| Protein peanut meal (Planter's), 2% | 50 |
| Distiller's dried solubles (Schenley Soludri), 3% | 38 |
| Linseed oil meal, 2% | 34 |
| Ground distiller's malt, 3% | 29 |

*The basal medium contained 2% brown sugar, 0.5% cornsteep liquor concentrate, and 0.1% $K_2HPO_4$.

When it was determined that highly purified preparations of the antibiotic contained over 10% sulfur, a large number of sulfur-

containing substances, both inorganic and organic, were added at different concentrations to the fermentation medium as potential precursors. These included sodium sulfate, sodium sulfite, sodium thiosulfate, sodium sulfide, cystine, cysteine, cysteic acid, glutathione, methionine, ethionine, 2-thioadenine, monothioglycerol, thiourea, thioacetamide, mercaptosuccinic acid, and many others. Unfortunately, none of these substances gave any significant yield increase. Assuming 0.47% sulfur in soy bean flour (actual determination), 14% sulfur in pure antibiotic X-5079C, and an activity of 300 units per mg of pure antibiotic, then it follows that if *all* the sulfur from the 2% soy flour in the fermentation medium were incorporated into antibiotic, it would permit a broth potency of only 210 units per ml. Actual broth yields of 50 units per ml therefore represent a 25% efficiency of conversion of soy flour sulfur to antibiotic sulfur. The streptomycete will grow in concentrations of its own antibiotic as high as 130 units per ml, so the concentration of antibiotic formed during fermentation does not appear to be the limiting factor.

The progress of a fermentation in a kettle is shown in Table 2. Peak yields are attained in 4 to 6 days, and, with the best strains of streptomycete, generally average about 40 to 50 units per ml. These strains were derived after considerable selection of spontaneous variants and selection from ultraviolet-irradiated survivors. The original strains produced only 5 to 10 units per ml. Although antibiotic production takes place from 24 to 32 C, it is best at 24 to 28 and much reduced at 30 to 32 C.

TABLE 2. *Antibiotic X-5079C production in a fermentation kettle**

| Fermentation time | pH | *P. varioti* units per ml, whole broth | Dry "cell" weight† |
|---|---|---|---|
| *days* | | | *mg/100 ml* |
| 0 | 6.6 | 0 | – |
| 1 | 6.9 | 0.8 | 275 |
| 2 | 7.2 | – | 528 |
| 3 | 7.4 | 26 | 553‡ |
| 4 | 7.4 | 35 | 373‡ |

*Carbon steel kettle K4, charged with 2,200 gallons of the following medium: 2% soybean flour, 2% brown sugar, 0.5% cornsteep liquor concentrate, 0.1% $K_2HPO_4$, and lard oil as antifoam. Temperature was 26 to 28 C; air flow was 50 cubic ft per min (expt. 4085); strain 3807-19A.

†These dry weights were obtained by acidifying duplicate 100-ml samples of broth to pH 3 and drying the filtered solids to constant weight at 110 C.

‡These dried solids contained 1.10 and 1.12% sulfur, respectively, compared with 0.47% in soy flour.

*Chemical isolation.* Antibiotic X-5079C may be extracted separately from mycelial growth and from the filtered broth, or, preferentially, the whole broth may be acidified with aluminum sulfate and phosphoric acid to pH 4. This accelerates filtration and precipitates the antibiotic almost quantitatively onto the mycelium-filter aid cake. The antibiotic may then be extracted from this with an alcohol such as butanol, using a volume about one-third that of the original whole broth. The clarified butanol extract is evaporated under reduced pressure below 45 C to about one-thirtieth of its original volume and precipitated with 2 volumes of petroleum ether. The precipitate is filtered off and air-dried to give a crude solid with about 30 *P. varioti* units per mg and a recovery of about 40% from the original whole broth.

The antibiotic may be further purified by treating the crude material in methanol with aluminum oxide, which adsorbs some impurities. Further purification can be achieved by extracting this product from a butanol solution with aqueous alkali (pH 7.5), acidifying the alkaline solution to pH 3.5, and extracting the antibiotic with butanol. Counter-current distribution between butanol and water (3 or 4:1) gave good purification results in the early work. The antibiotic can also be purified by chromatography in methanol on a column of basic aluminum oxide. Instead of using aluminum oxide, the mixture may be purified by chromatography on a silica gel distribution column or a water-containing diatomaceous earth ("Hyflo super-cel," Johns-Manville) column, using butanol saturated with water as the mobile phase. For the preparation of highly purified materials, various combinations of two or more of the above steps have been successfully employed (Table 3).

TABLE 3. *Antibiotic yields during isolation*

| Purification step | Total units in millions | Yield of dry solids | Units/mg purity | Yield |
|---|---|---|---|---|
| | | *kg* | | % |
| 2,000-gallon broth | 330 | – | 1-2 | 100 |
| Petroleum ether precipitate of butanol extract | 140 | 4.7 | 25-35 | 43 |
| Alumina batch treatment | 113 | 2.2 | 50-55 | 34 |
| Partition, butanol-acid-alkali | 80 | 0.94 | 80-90 | 24 |
| Hyflo-butanol-water distribution column | 33 | 0.22 | 140-160 | 10 |
| Alumina column chromatography | 14 | 0.055 | 240-280 | 4* |

*This yield could, of course, be increased to some extent by subsequent reworking of side fractions.

The following is a detailed description of one isolation procedure: 3 kg of crude antibiotic X-5079C (25 to 35 *P. varioti* units/mg) were dissolved in 35 liters of methanol. To this solution, 6 kg of aluminum oxide XF 21 (Alcoa) were added, and the mixture was stirred

for 30 min. It was then filtered and the filter cake was washed with 5 liters of methanol. The combined filtrates were concentrated in vacuo to 6 liters and diluted with 18 liters of a 1:1 mixture of acetone-Skellysolve B. The precipitated material was filtered off. It formed 1.4 kg of light brown solids with an in vitro activity of 50 to 55 units/mg. This material was mixed with 7 liters of water and 7 liters of butanol. The mixture was stirred, and the pH was adjusted to 7.5 by addition of 3 N sodium hydroxide. The water layer was then separated and the butanol layer was extracted three more times with 2 liters of water each time. The aqueous layers containing most of the antibiotic were combined, acidified to pH 3.5 with 75% phosphoric acid, and extracted once with 7 liters and then three times with 3 liters of butanol. The combined butanol extracts were neutralized (pH 7) with dilute aqueous sodium hydroxide and concentrated in vacuo to about 12 liters. To this concentrate were added 24 liters of a 1:1 mixture of acetone and Skellysolve. The precipitated tan powder (600 g, 80 to 90 units/mg) was filtered off. This material (600 g) was dissolved in 1 liter of water and mixed with 1.5 kg of diatomaceous earth (Hyflo super-cel). The moist powder thus obtained was suspended in about 3 liters of water-saturated butanol and put on top of a distribution column which consisted of a mixture of 4.5 kg of diatomaceous earth (Hyflo super-cel) and 3 liters of water made up with water-saturated butanol. The column was eluted with about 100 liters of water-saturated butanol. The eluate was collected in fractions of about 2 liters. Each fraction was tested microbiologically. Finally, fractions (5 to 10) of about equal potency were combined and concentrated in vacuo to a small volume. During the concentration some of the material precipitated out. An excess of petroleum ether was added to the concentrates. The material was precipitated as a fine tan powder, which was filtered off and washed with petroleum ether. Thus a number of fractions were obtained, some of which were combined, and yielded finally about 140 g of material of a potency of 140 to 160 units/mg, about 100 g of 50 to 70 units/mg, and about 150 g of 0 to 20 units/mg. The further purification of the material was carried out as follows: 80 g of the peak fractions (140 to 160 units/mg) were dissolved in 2 liters of methanol and put on a column, prepared with methanol and 1.2 kg of basic aluminum oxide (Woelm or Merck) and deactivated by the addition of water to 15%. Methanol was used for the elution; 1-liter fractions were taken, concentrated in vacuo, and then precipitated with acetone-petroleum ether. The first fractions yielded the best material; 15 to 20 g of a potency of 240 to 280 units/mg and about 10 g of 150 to 200 units/mg were recovered as an almost white powder. A 2% solution of the best material (240 to 280 units/mg) in water had a pH of 8.2.

Instead of using aluminum oxide in the last step of purification, the material may also be purified by chromatography on a silica gel

distribution column. This column was prepared as follows: To a mixture of 1 kg of Davison silica gel (100 to 200 mesh) and 300 g of Hyflo were added 400 ml of the *lower* phase of a mixture of 19 parts by volume butanol, 1 part by volume petroleum ether, and 5 parts by volume water. This moistened solid was then suspended in the necessary amount of the *upper* phase, and transferred into a chromatographic tube. The material to be purified was dissolved in water (15 g of an activity of about 200 units/mg in 50 ml of water) and mixed with 120 g of silica gel and 36 g of Hyflo. This mixture was suspended in the necessary amount of the upper phase of the butanol-petroleum ether-water mixture and transferred onto the top of the column. The material was then eluted with the same mixture. Then 1-liter fractions were collected, and those showing the same activity were combined and concentrated in vacuo to a small volume. The precipitated material was then filtered off and washed with petroleum ether. Thus, 5.4 g of material of about 300 units/mg and 6.9 g of 100 to 200 units/mg were isolated as beige powders. The best material (300 units/mg) gave a turbid 0.1% solution in water with a pH of 3.6.

*Chemical and physical properties of antibiotic X-5079C.* The best preparations of the antibiotic are amorphous white powders which have an activity of 250 to 300 *P. varioti* units/mg. They have no distinct melting points, darken at 220, brown at 250, and char at 270 to 280 C. Purified preparations show the following elementary analyses: C, 46 to 48; H, 4 to 5; N, 14 to 16; S, 12 to 14; and Na 1 to 2. The spread in the analysis results is probably due to the various degrees of purity of the material. The primary measure for the purity is the in vitro bioassay, which at the very best is reliable only to $\pm$10%. In addition, samples prepared by different purification procedures contained varying ratios of free carboxylic acid to sodium salt, which accounts for the variations in the sodium content and might also be responsible for different degrees of hydration. Samples of lower microbiological activity generally show a lower sulfur content. It is interesting to compare these analyses with those reported by Sensi, Ballotta, and Gallo (1958) for the antibacterial antibiotic matamycin: C, 43.95; H, 4.06; H, 14.45; S, 13.57; $[\alpha_D] = +36.6^{\circ}$. Yamaguchi et al. (1957) reported for the antibacterial antibiotic althiomycin (now believed to be identical with matamycin) the analyses C, 44.49; H, 3.51; N, 13.92; S, 14.77.

The specific optical rotation $[\alpha]_D^{25}$ is + 40 to 42° (c = 1.0 in water). When acidified (pH 3 to 4), the antibiotic is fairly soluble in methanol and water-saturated butanol. It is only slightly soluble in water and acetone, and is insoluble in ether, ethyl acetate, chloroform, benzene, and hexane. At pH values between 7 and 9, the antibiotic is soluble in methanol and water (the sodium salt will give a 10% aqueous solution), only slightly soluble in butanol, insoluble in ether, ethyl acetate, chloroform, benzene, acetone, and hexane. The compound is also soluble in pyridine.

Antibiotic X-5079C shows maxima in the ultraviolet absorption spectrum in isopropanol at 222 mμ, $E^{1\%}_{1\ cm}$ = 510, and at 273 mμ, $E^{1\%}_{1\ cm}$ = 245 (Fig. 1). The infrared absorption spectrum of this antibiotic in KBr is reproduced in Fig. 2.

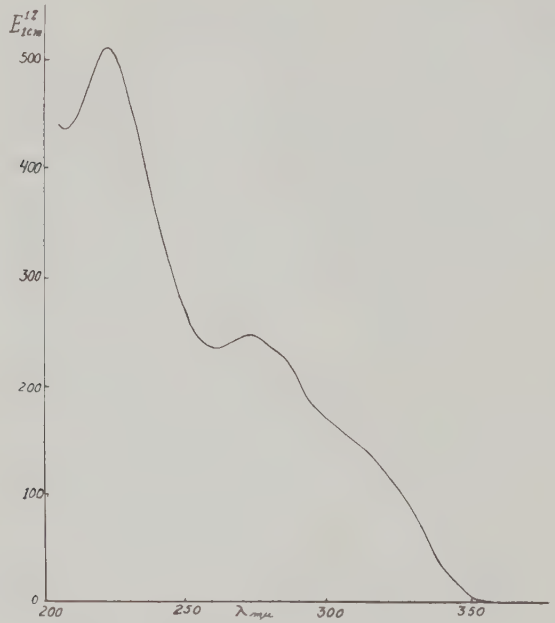


FIG. 1. *Ultraviolet absorption spectrum of antibiotic X-5079C in isopropanol.*

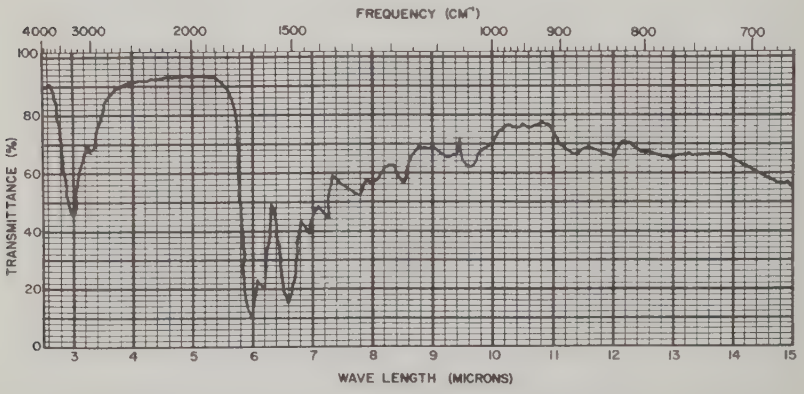


FIG. 2. *Infrared absorption spectrum of antibiotic X-5079C in KBr.*

Aqueous solutions of antibiotic X-5079C are fairly stable at low temperatures at a pH between 4 and 8. The microbiological activity disappears in strongly acidic and strongly basic solutions.

Since the antibiotic does not readily dialyze through cellophane, it must be of high molecular weight, probably over 3,000. The in vitro active material excreted in the urine of monkeys treated subcutaneously is still not readily dialyzable through cellophane, which indicates that the excreted antibiotic is of high molecular weight even after its passage through the animal. (Actually some 14% of the urine activity and 7% of the activity in a control purified antibiotic solution appear to have dialyzed after 66 hr.) Emmons and Piggott (1961) also indicated that incubation of the antibiotic with sterile fresh mouse tissue does not convert the substance into a more active form that could be detected by in vitro test.

The antibiotic can be hydrolyzed by refluxing with 20% hydrochloric acid. Treatment of the hydrolyzate with 30% hydrogen peroxide, to convert any cysteine or cystine into cysteic acid, and then subjecting the solution to bidimensional chromatography on paper, using phenol-water (100:25) in a medium saturated with ammonia and collidine-lutidine-water (1:1:1) in a medium saturated with diethylamine, gives eight ninhydrin-positive spots. Five of these could be identified as due to cysteic acid, aspartic acid, glycine, threonine, and proline. This indicates that the antibiotic is a polypeptide of high molecular weight.

*Microbiological properties.* Antibiotic X-5079C is particularly distinguishable because of its very narrow in vitro antimicrobial spectrum. It is inactive on agar-plate test against the yeasts *Saccharomyces cerevisiae, S. carlsbergensis, Kloeckera brevis, Candida albicans, Torula cremoris, T. utilis, T. rosea, Hansenula saturnus, H. anomola, Pichia chodata, P. membran aesaciens, Zygosaccharomyces saturnus, Z. barkeri, Z. bisporus, Schizosaccharomyces pombé, S. octosporus, Debaryomyces tyrocola,* and *D. globosus.* Although a number of other fungi, in addition to *P. varioti,* show sensitivity in vitro to the new antibiotic on agar assay plates, none of those tested *(Penicillum digitatum, P. citrinum, Aspergillus flavus, A. niger, Alternaria solani, Fusarium oxysporum, Rhizopus nigricans, Botrytis paeoniae, Helminthosporium inequale, Histoplasma capsulatum)* is nearly as sensitive as *P. varioti,* nor do they give as satisfactory inhibition zones.

Antibiotic X-5079C is inactive in agar cup-plate tests against the following bacteria: *Escherichia coli, Mycobacterium phlei, Bacillus simplex, Sarcina lutea, Corynebacterium simplex, Streptomyces cellulosae, B. subtilis, Azotobacter vinelandii, Staphylococcus aureus,* Bodenheimer's bacillus, *Proteus vulgaris, Pseudomonas aeruginosa, P. ovalis, Alcaligenes faecalis, Aerobacter aerogenes, Lactobacillus lactis, B. adhaerans, B. cereus, B. megaterium, S. albus,* and *Streptococcus faecalis.*

*Chemotherapeutic properties.* Antibiotic X-5079C was found to be active against histoplasmosis in mice, with a $CD_{50}$ of 2 to 5 mg per kg per day, subcutaneously, and against *Blastomyces dermatitidis* infections in mice, with a $CD_{50}$ of 8.5 mg per kg per day, subcutaneously. Its $LD_{50}$ in mice is 2,000 mg/kg, subcutaneously; 707 mg/kg, intravenously; and 2,000 mg/kg, intraperitoneally. Recent publications (Grunberg et al., 1961; Emmons, 1961; Utz et al., 1961) have described extensions of these findings in animals and in man.

## Discussion

A new microorganism, *Streptomyces* sp. n. X-5079, has been found to produce a new antifungal antibiotic. This acidic substance, occurring mostly in the mycelium, is characterized by a very narrow in vitro antimicrobial spectrum, a high molecular weight, polypeptide nature, high sulfur content, high solubility in water of its sodium salt, and low toxicity in mice coupled with high systemic in vivo activity against *Histoplasma capsulatum*. Yields in the fermentation broths and on chemical isolation are low.

## Acknowledgments

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## Literature Cited

Andriole, V. T., J. P. Utz, and S. M. Sabesin. 1961. Altered sulfobromophthalein metabolism in man induced by the antifungal agent X-5079C. Am. Rev. Respiratory Diseases **84**:538-548.

Emmons, C. W. 1961. Chemotherapeutic and toxic activity of the antifungal agent X-5079C in experimental mycoses. Am. Rev. Respiratory Diseases **84**:507-513.

Emmons, C. W., and W. R. Piggott. 1961. Bioassay of the antifungal agent X-5079C in serum of patients. Am. Rev. Respiratory Diseases **84**:534-537.

Grunberg, E., J. Berger, and E. Titsworth. 1961. Chemotherapeutic studies on a new antifungal agent, X-5079C, effective against systemic mycoses. Am. Rev. Respiratory Diseases **84**: 504-506.

Louria, D. B. 1961. The treatment of experimental fungus infections. Chemotherapia **3**:95-107.

Sensi, P., R. Ballotta, and G. G. Gallo. 1958. Matamycin, a new antibiotic. II. Isolation and characterization. Antibiotics & Chemotherapy **9**:76-80.

Utz, J. P., V. T. Andriole, and C. W. Emmons. 1961. Chemotherapeutic activity of X-5079C in systemic mycoses of man. Am. Rev. Respiratory Diseases **84**:514-528.

Yamaguchi, H., Y. Nakayama, K. Takeda, and K. Tawara. 1957. A new antibiotic, althiomycin. J. Antibiotics (Japan) **10A**:195-200.

# A CRYSTALLINE ANTIBIOTIC FROM A THERMOPHILIC STREPTOMYCETE

## I. Biological Properties

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The search for new antibiotics has led workers to study unique groups of microorganisms. During the past few years one group of microbes carefully considered by us has been the thermophilic actinomycetes. One of the cultures isolated at 50 C in this search was found to produce a new, broad-spectrum antibiotic, 19A. This paper and the one to follow by Putter and Wolf (1962) describe in detail the isolation, fermentation, purification, and chemical properties of 19A.

### Materials and Methods

The organism which produces 19A, culture MA-568, was obtained from a Jamaica, B.W.I., soil plated out in the conventional manner on a dextrose-asparagine-yeast (DAY) medium (Table 1). Petri plates

TABLE 1. *Media used for growth of culture MA-568*

| DAY | | SBM | | NYD | |
|---|---|---|---|---|---|
| % | Compound | % | Compound | % | Compound |
| 1.0 | Dextrose | 3.0 | Extracted soybean meal[b] | 0.3 | Beef extract[a] |
| 0.1 | D-Asparagine | 2.0 | Cerelose[c] | 0.5 | Peptone[a] |
| 0.01 | $K_2HPO_4$ | 0.75 | Distiller's solubies[d] | 0.5 | Yeast extract[a] |
| 0.05 | $MgSO_4 \cdot 7H_2O$ | 0.25 | NaCl | 1.0 | Dextrose |
| 0.001 | $FeSO_4 \cdot 7H_2O$ | | | | |
| 0.05 | Yeast extract[a] | | | | |
| pH adjusted to 7.2 before sterilization | | pH adjusted to 7.5 before sterilization | | | |

[a] Difco Laboratories, Detroit, Mich.
[b] Archer-Daniel-Midland, Minneapolis, Minn.
[c] Hiram Walker & Sons, Ltd., Walkerville, Ontario, Canada.
[d] Corn Products Refining Co. Argo, Ill.

were enclosed in tin cans with close-fitting lids to curtail moisture loss, and were incubated at 50 C for 2 to 4 days. Activity was first noted by spraying the isolation plate with a suspension of *Escherichia coli* W cells in 0.8% KCl, incubating the plate at 25 C for 18 hr, and then measuring inhibition zones around actinomycete colonies which produced active principles.

The active colony then was transferred by loop to form a streak on a DAY agar plate and incubated for 2 days at 50 C. By utilizing several *E. coli* mutants made resistant to a series of antibiotics (Stapley, 1958), no cross-resistance pattern of a known antibiotic was observed after antibiotic-resistant mutants had been cross-streaked up to MA-568 and incubated at 25 C for 18 hr.

DAY agar slant cultures were incubated at 50 C for 2 to 4 days, after which they were stored at room temperature until used. The culture also was lyophilized for more permanent storage.

Submerged fermentations were carried out by the usual shaker-flask method, in glass 5-liter fermentors, and in 150-gal fermentors. Inoculum was prepared by suspending the spores from one slant into 10 ml of sterile 0.8% KCl and adding 2 ml of this suspension to 50 ml of vegetative (NYD) medium (Table 1) in a 250-ml Erlenmeyer flask. After incubation at 50 C on a 2-in.-stroke rotary shaker (220 rev/min) for 24 to 48 hr, the resultant broth was added to DAY or SBM production media (Table 1) at a 10% (v/v) level. For large-scale fermentations, the general procedure was the same except that larger batches of inoculum were made.

## Results

Culture MA-568 has been compared to the four species of thermophilic streptomycetes described in Bergey's Manual (Breed, Murray, and Smith, 1957) and to the thermophilic microorganisms described by Waksman (1961). The microorganism is a member of the genus *Streptomyces*. It differs significantly from *S. rectus* Henssen and *S. thermofuscus* nov. comb., which are the only thermophilic species of *Streptomyces* which produce soluble brown pigments on organic media.

*Morphology.* Sporophores straight with short conidial chains. Spores spherical, 1.0 $\mu$ in diameter, dark olive.

*Skim milk.* Milk peptonized. Dark ring, gray sediment. Milk darkened, almost black. Reaction alkaline around pH 7.2.

*Litmus milk.* Milk peptonized, no ring formed. Spores gray, floating on surface of tube. Milk turned brown. Reaction neutral.

*Gelatin.* Liquefication (30%) in 3 weeks. Dark brown to almost black soluble pigment in medium. Spores cream-colored. Abundant cream-colored aerial mycelium floating on the surface of liquefied gelatin.

*Nitrate agar.* Medium turns light brown. Aerial mycelium and spores cream-colored. Nitrates not reduced to nitrites.

*Hydrogen sulfide medium.* Good vegetative growth on Difco peptone iron agar medium. Aerial mycelium and spores bluish gray. Medium blackened ($H_2S$ test positive). Reverse black.

*Tyrosine agar.* Good vegetative growth. Aerial mycelium and spores gray. Reverse unchanged.

*Ca-malate agar.* Fair growth. Raised colonies. Gray aerial mycelium and spores. Calcium malate not digested.

*Czapek-Dox agar.* Numerous small round colonies. Gray in color. Good aerial mycelium and spore growth. No soluble pigment. Reverse not changed. Small colonies radiate from a central point in a circular manner.

*Oatmeal agar.* Very good vegetative growth. Colonies coalesce to form a uniform surface growth with numerous spores and aerial mycelia. Colonies powdery. No soluble pigment. Reverse brown.

*Citrate agar.* Good vegetative growth. Aerial mycelium and spores almost white (very light grayish tint). Reverse unchanged. No soluble pigment.

*Potato plug.* Good vegetative growth. Colonies wrinkled and sunk in the center with raised margins. Abundant aerial mycelium and spores, white to gray in color. Medium brown.

*Dextrose-asparagine agar.* Very good vegetative growth. Abundant aerial mycelium and spores. Spores gray in color. Colonies powdery. Medium slightly brown (light brown soluble pigment).

*Sabouraud maltose agar.* Good vegetative growth. Aerial mycelium and spores yellowish-gray. Soluble pigment black, coffee-colored. Reverse black, coffee-colored. Surface powdery.

*Emerson agar.* Very good vegetative growth. Cream-colored aerial mycelium and spores. Reverse light to dark brown.

*Nutrient agar.* Scant growth. Aerial mycelium and spores light cream-colored. Reverse light brown.

*Bennett agar.* Excellent growth. Numerous spores, giving the surface of growth a very powdery appearance. Light brown soluble pigment. Reverse light to dark brown. Dark gray spores.

*Starch agar.* Good vegetative growth. Aerial mycelium and spores white. Surface of growth powdery. No soluble pigment. Reverse very light brown. Starch is not hydrolyzed.

Fermentation broths were evaluated, by paper-disc ($\frac{1}{2}$-in. diameter) assay and tube dilution assay (Difco phenol red broth) vs. *E. coli* W, from 18 hr through 6 days of incubation at 50 C. Initially, no inhibition was noted unless the broth was concentrated 2- to 10-fold. The 2 × concentrate gave a zone of 17 mm; at 10 × the zone was 24 mm. A cross-resistance pattern of the concentrate indicated that the chloramphenicol-resistant mutant of *E. coli* and, to a lesser degree, the viomycin-resistant mutant were unresponsive to the active broth (Fig. 1). After the antibiotic had been isolated and crystallized,

this pattern was verified: reduced activity for chloramphenicol- and viomycin-resistant cultures and a supersensitivity response by the neomycin-resistant culture. The SBM medium supported slightly better production.

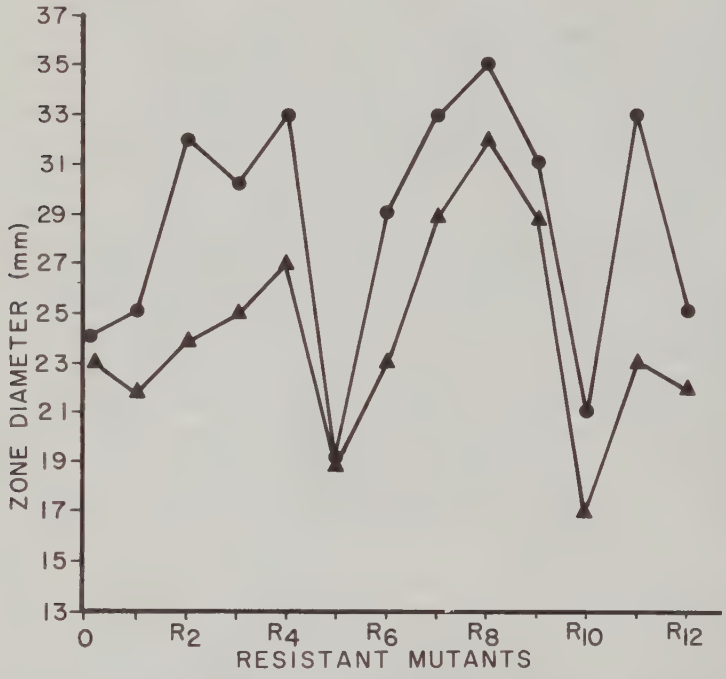


FIG. 1. *Cross-resistance patterns of antibiotic 19A broth and crystalline antibiotic 19A. ●: filtered broth concentrated tenfold; ▲: crystalline antibiotic 19A, 100 μg/ml. Escherichia coli and mutants: 0, sensitive parent; $R_1$, streptomycin; $R_2$, streptothricin; $R_3$, cycloserine; $R_4$, pleocidin; $R_5$, chloramphenicol; $R_6$, chlortetracycline; $R_7$, oxytetracycline; $R_8$, neomycin; $R_9$, tetracycline; $R_{10}$, viomycin; $R_{11}$, polymyxin B; and $R_{12}$, grisein.*

Further fermentation trials showed a general loss in the amount of activity produced at higher incubation temperatures, especially if incubation lasted more than 48 hr. However, by dropping the temperature for production in SMB medium to 28 C and by using 24-hr-old vegetative inoculum, the activity was not lost as rapidly.

It was noted also that the pH values dropped considerably from the initial 6.8 to less than 5.0 in the first 24 hr, and then rose to higher than 8.0 in the next 12 to 24 hr (Fig. 2). By controlling pH throughout the fermentation, higher activity could be assured. This was done by using glass 5-liter fermentors with automatic equipment

to add alkali (10 N NaOH), if broth pH dropped below 6.7, or acid (6 N HCl), if the pH went above 7.2. The difference between levels of activity of the controlled pH vs. uncontrolled pH fermentations is apparent.

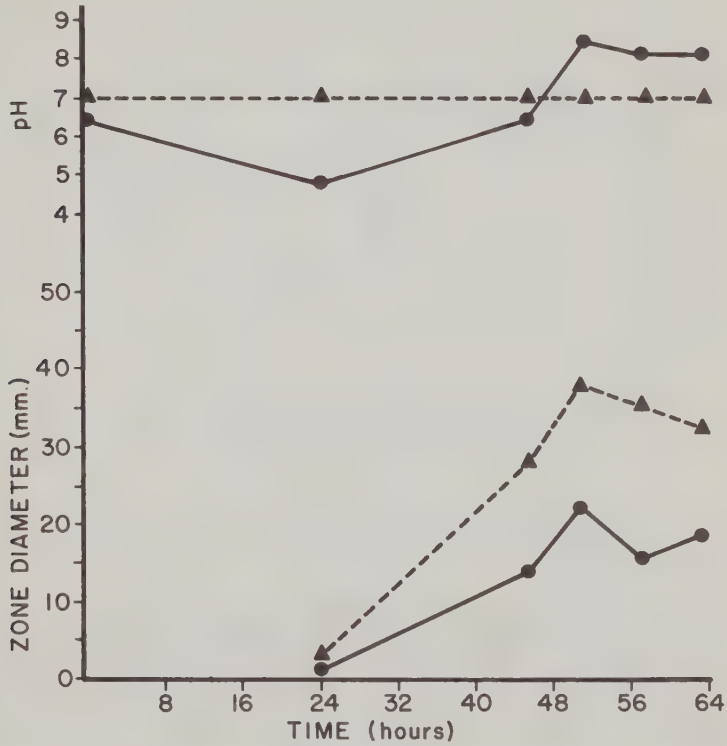


FIG. 2. *Relationship of pH and antibiotic 19A production during fermentation at 28 C. ●: pH noncontrolled, filtered broth concentrated tenfold; ▲: pH controlled, filtered broth concentrated tenfold.*

During this time, we noted that, by use of controlled pH and selection of a white-spored subculture of MA-568, we would be assured of a broth containing an increased level of antibiotic 19A, although other antibiotics would still be detected. In the absence of pH control, many of the varied morphological subcultures of culture MA-568 produced fermentation broths in which the cross-resistance assay pattern was not similar to that of 19A. The presence of other antibiotics caused a different cross-resistance response (Fig. 3). Furthermore, extracted spent broth from noncontrolled fermentations contained active materials dissimilar to 19A. The production of two or

more antibiotics by culture MA-568 has been confirmed many times since, and puts the culture into the same category as many mesophilic microbes which produce mixtures of antibiotics.

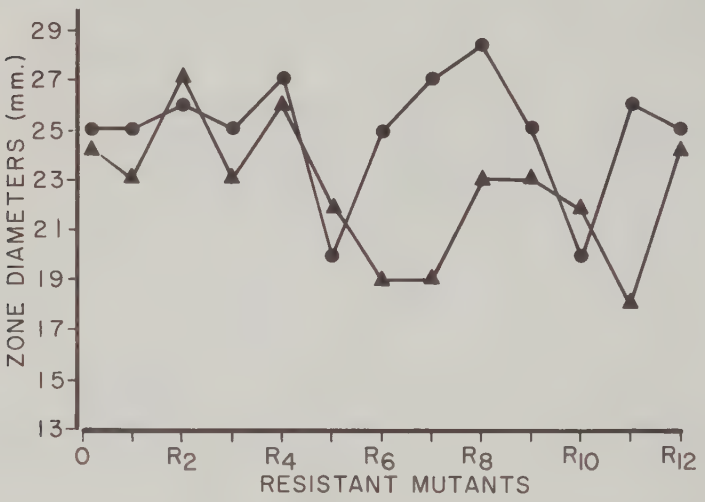


FIG. 3. *Cross-resistance patterns of pH controlled and noncontrolled antibiotic 19A fermentations.* ●: *pH controlled at 7.0, 48-hr harvest, filtered broth;* ▲: *pH uncontrolled, 48-hr harvest, filtered broth concentrated tenfold.*

Biospectrum assays for crystalline 19A were obtained; 5 ml of Difco nutrient agar and 0.2% yeast extract were used in petri plates. The inoculum was 5 ml per 150 ml agar of an overnight (37 C) nutrient broth culture which had been adjusted to a standard cell density on a Lumetron colorimeter (Will Corp., Buffalo, N.Y.) using a 660 mμ filter. Incubation of these assay plates was at 25 C for 18 to 24 hr.

Table 2 gives the results of such assays with crystalline antibiotic 19A. The antibiotic is an acid, as described by Putter and Wolf (1962), and, as such, loses its activity in the alkaline range. Its breadth of bioactivity removes it from the so-called narrow-spectrum or gram-positive active group of antibiotics. Because of its relatively low molecular weight (330), it has a high order of diffusibility, as illustrated by the increase of 2.5 to 4.0 mm in diameter of the zone of inhibition in an agar diffusion assay for each doubling of concentration. Its minimal inhibitory concentration vs. *E. coli* in the tube dilution assay is 4 μg/ml.

Experimental infections in mice, with *Salmonella schottmuelleri* as the infective agent, were treated with antibiotic 19A. Table 3 shows that some protection was afforded mice so treated. The sum

of the two affecting agents in the mice (treatment with 19A at about one-tenth the toxicity level and the infective bacteria) probably caused death at dosage levels slightly above the $ED_{50}$.

TABLE 2. *In vitro antimicrobial spectrum of crystalline antibiotic 19A*

| Organism | Concn | Agar diffusion zone diameter[a] |
|---|---|---|
| | *μg/ml* | *mm* |
| *Diplococcus pneumoniae* | 100 | 16 |
| *Staphylococcus aureus* | 100 | 23 |
| *Bacillus subtilis* | 100 | 29 |
| *Sarcina lutea* | 100 | 30 |
| *Klebsiella pneumoniae* | 100 | 14 |
| *Salmonella schottmuelleri* | 100 | 15 |
| *Pseudomonas aeruginosa* | 100 | 19 |
| *Serratia marcescens* | 100 | 19 |
| *Proteus vulgaris* | 100 | 29 |
| *Escherichia coli* W (pH 5,7, 9) | 100 | 32, 22, 0 |
| *E. coli* | 250 | 26 |
| *E. coli* | 500 | 30 |

[a]The pH was 7.0 unless otherwise indicated.

TABLE 3. *Activity of antibiotic 19A in experimental infections in mice*[a]

| Test | Dose | Survivors/total | Mean survival time | $ED_{50}$ | Tolerated dose[b] |
|---|---|---|---|---|---|
| | *mg* | | *days* | *mg/dose* | *mg* |
| 1 | 0.0 | 0/5 | 0.9 | | |
| | 0.125 | 5/5 | 7.0 | <0.125 | >1.0 |
| | 0.25 | 1/5 | 2.2 | | |
| | 0.5 | 1/5 | 2.8 | | |
| 2 | 0.0 | 0/5 | 0.7 | | |
| | 0.0625 | 0/5 | 1.4 | 0.115 | <2.0 |
| | 0.125 | 3/5 | 4.6 | | >1.0 |
| | 0.25 | 0/5 | 1.6 | | |

[a]Infection: *Salmonella schottmuelleri,* Route: intraperitoneal. Mean survival time: 0.8 days. Therapy: intraperitoneal treatment at 0, 6, and 24 hr.

[b]Uninfected mice.

## Discussion

Rode, Foster, and Schuhardt (1947) reported penicillin production by a thermophilic fungus, *Malbranchia pulchella.* Maximal activity

of 5 IU/ml of broth was obtained in 4 days at 52 C and in 13 days at 37 C.

Burton (1950) described a quinonoid compound, thermophillin, produced by a polyporaceous basidiomycete from the tropics. It was active against *Staphylococcus aureus* in vitro.

Schöne (1951) reported on a systematic screening of thermophilic actinomycetes from soil, milk, and tooth tartars. He found a strain of *Streptomyces thermophilus* which produced thermomycin. The antibiotic was produced at temperatures of 30 to 60 C in 2 to 14 days and was active against a *Corynebacterium* sp. at rather high broth dilutions (1:256). It was a stable material but inactive vs. *S. aureus* and other gram-positive or gram-negative bacteria.

Aiso et al. (1952) reported on another antibiotic screening of thermophiles, among which two antibiotic producers were found. The first was A25, which produced a litmocidin-like compound at 45 to 50 C in Czapek's medium and was active in vitro against *S. aureus*. The second was A26, which produced a butanol-soluble (pH 8.0) antibiotic that could be recovered as an amorphous yellow powder and was active in vitro against *S. aureus*. Incubation was at 50 to 60 C, with feeble growth and no production at 37 C.

More recently, Schuurmans, Olson, and San Clemente (1956), reported on the isolation of thermoviridin from an actinomycete growing at 55 C. The acidic antibiotic, produced only after prolonged inoculum build-up, was primarily active against gram-positive bacteria.

This report on 19A is the first record in the literature of the discovery, isolation, and crystallization of an antibiotic produced by a thermophilic actinomycete. Although culture MA-568 is not an obligate thermophile, it was isolated and detected as an antibiotic producer at a high temperature. Several of the organisms described in the literature referred to above as thermophiles also could more properly be called facultative thermophiles after the classification in the 5th ed. of Bergey's Manual (Bergey et al., 1939). These cultures, as well as 19A, are characterized by development at 28 C. optimal growth at 50 C, and a maximal tolerated temperature of 60 C. True thermophiles have their optimum at 60 to 70 C, with little or no growth below 40 to 50 C.

### Acknowledgments

We are greatly indebted to A. Kathrine Miller of the Merck Institute for Therapeutic Research who performed the therapeutic assays against experimental infections in mice.

## Literature Cited

Aiso, K., T. Arai, T. Hashimoto, I. Shidara, and K. Ogi. 1952. Thermophilic actinomycetes as antibiotic producers. Rept. Inst. Putrefaction Research, Chiba Univ., Japan 5:100-107.

Bergey, D. H., R. S. Breed, E. G. M. Murray, and A. P. Hitchens. 1939. Bergey's manual of determinative bacteriology, 5th ed. The Williams & Wilkins Co., Baltimore.

Breed, R. S., E. G. B. Murray, and N. R. Smith. 1957. Bergey's manual of determinative bacteriology, 7th ed. The Williams & Wilkins Co., Baltimore.

Burton, H. 1950. An antibiotic, thermophillin, from *Lenzites thermophilia*. Nature 166:570.

Putter, I., and F. J. Wolf. 1962. A crystalline antibiotic from a thermophilic streptomycete. II. Isolation and chemical properties. Antimicrobial Agents and Chemotherapy - 1961, p. 454-461.

Rode, L. J., J. W. Foster, and V. T. Schuhardt. 1947. Penicillin production by a thermophilic fungus. J. Bacteriol. 53:565-566.

Schöne, R. 1951. An antibiotic which inhibits *Corynebacterium diphtheriae* produced by the S form of *Streptomyces thermophilus*. Antibiotics & Chemotherapy 1:176-180.

Schuurmans, D. M., B. H. Olson, and C. L. San Clemente. 1956. Production and isolation of thermoviridin, an antibiotic produced by *Thermoactinomyces viridis* n. sp. Appl. Microbiol. 4: 61-66.

Stapley, E. O. 1958. Cross-resistance studies and antibiotic identification. Appl. Microbiol. 6: 392-398.

Waksman, S. A. 1961. The actinomycetes, vol. 2. The Williams & Wilkins Co., Baltimore.

# A CRYSTALLINE ANTIBIOTIC FROM A THERMOPHILIC STREPTOMYCETE
## II. Isolation and Chemical Properties

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The conditions for the production of antibiotic 19A by fermentation, together with the in vivo and in vitro properties of this antibiotic, have been described by Miller, Barreto, and Woodruff (1962). This report will describe the isolation and chemical properties of the antibiotic.

Since antibiotic activity present in the early fermentation broths was very low, attempts to determine the chemical properties of the antibiotic were hampered by the necessity of concentrating the broth for assay. It was not until a pH-controlled fermentation was developed that progress was made in determining the chemical properties of the antibiotic. Antibiotic 19A refers to the solvent-soluble acidic substance produced by the culture. Chemical evidence for the simultaneous production of another substance with antibiotic activity was obtained.

A chemical screening procedure indicated that antibiotic 19A was not adsorbed at neutral pH on either a carboxylic or sulfonic acid ion-exchange resin. It was adsorbed on a porous quaternary exchange resin, but not on a weak-base anion resin. The active principle was extractable under acidic conditions from broth filtrates with amyl acetate, methyl isobutyl ketone, butanol, and ethyl acetate. Extraction with ethyl acetate was less favorable at neutral pH. This indicated the acidic nature of the antibiotic. The antibiotic in broth was dialyzable, indicating a relatively low molecular weight compound. The antibiotic was adsorbed on charcoal. Elution, however, was poor under various conditions tried.

### Experimental

The effect of pH on the stability of antibiotic 19A was determined by adjusting broth filtrates to pH 3.0, 7.0, and 10.0 with hydrochloric acid and sodium hydroxide. These samples were stored at room temperature for various time intervals and then neutralized

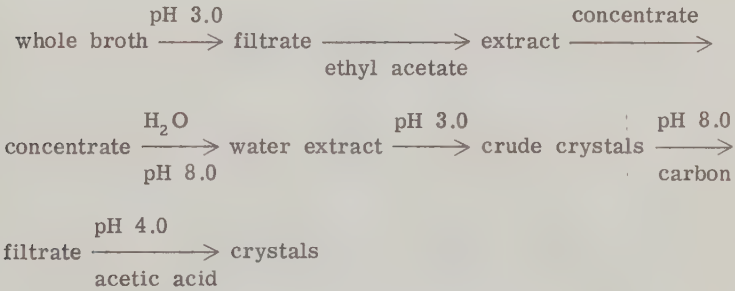
to pH 7.0 for assay (Table 1). It is apparent that there was a small loss of activity under acidic conditions, but rapid inactivation at neutral and alkaline pH's. Since there was little change in the antibiotic activity after 1 hr at pH 10.0, a second alkali-stable antibiotic may be present.

TABLE 1. *Stability of antibiotic 19A broth**

| | Hours at 25 C | | | | |
|---|---|---|---|---|---|
| pH | 0 | 1 | 2 | 24 | 48 |
| 3.0 | 20 | 20 | 20 | 19 | 18.5 |
| 7.0 | 20 | 20 | 19 | 16 | 14 |
| 10.0 | 19 | 15 | 15 | 15 | 14 |

*Results are expressed as the diameter (mm) of the zones of inhibition, with *Escherichia coli* as the test organism.

Because of the exact conditions required for the production of antibiotic 19A, and its instability at pH's existing in the fermentation, broth potencies rarely exceeded 50 μg/ml. This necessitated production in large fermentation vessels to support the isolation studies. The procedure used for the isolation of antibiotic 19A was the following:




Harvested whole broth was immediately acidified to pH 3.0 and the mycelium removed with the use of filter aid. The filtrate was extracted twice with equal volumes of ethyl acetate. The solvent extract was concentrated approximately fiftyfold in vacuo. The active fraction was back-extracted into water at pH 8.0, and then crystallized by the gradual addition of acid, with stirring, to pH 3.0. The free acid was recrystallized from hot water, methanol, or ethyl acetate.

Table 2 summarizes the distribution coefficients of antibiotic 19A as a function of pH. Of the solvents studied, ethyl acetate was the solvent of choice since butanol and amyl acetate did not give as favorable distribution ratios into the solvent.

TABLE 2. *Distribution coefficients of antibiotic 19A*

| pH | System* | K solvent/water |
|---|---|---|
| 2.0 | Phosphate ethyl acetate | 2.9 |
| 3.0 | Phosphate ethyl acetate | 2.6 |
| 4.0 | Phosphate ethyl acetate | 1.3 |
| 5.0 | Phosphate ethyl acetate | 0.34 |
| 6.0 | Phosphate ethyl acetate | 0.17 |
| 7.0 | Phosphate ethyl acetate | 0.06 |
| 8.0 | Phosphate ethyl acetate | 0.01 |
| 3.0 | Phosphate butanol | 1.1 |
| 3.0 | Phosphate amyl acetate | 1.2 |

*All at 0.1 M.

The paper chromatographic behavior of antibiotic 19A in an ethyl acetate solvent system was studied at various pH levels (Table 3). A downflow system with buffer, as stationary phase, and Whatman no. 1 paper was employed. Active zones were visualized by the bioautographic technique. It should be noted that two components were present in broth samples, thus confirming the presence of a second antibiotic.

TABLE 3. *$R_F$ values for antibiotic 19A in an ethyl acetate-0.1 M phosphate buffer system*

| pH | Broth | Crystals |
|---|---|---|
| 3.0 | 1.0<br>0.10 | 1.0 |
| 5.0 | 1.0<br>0.10 | 1.0 |
| 6.0 | 0.69<br>0.10 | 0.69 |
| 7.0 | 0.70<br>0.10 | 0.70 |
| 8.0 | 0.10 | 0.08 |
| 9.0 | 0.00 | 0.00 |

Table 4 summarizes additional $R_F$ values in other solvent systems, which tend to distinguish this antibiotic from any of the knowns.

TABLE 4. *$R_F$ values of antibiotic 19A in various solvent systems*

| System | $R_F$ |
|---|---|
| Isopropanol-water (70:30) | 0.54 |
| Sodium chloride-water-methanol (2.0%:25:75) | 0.79 |
| Butanol-0.1 M phosphate (pH 7.0) | 0.08 |
| Methyl isobutyl ketone-0.1 M phosphate (pH 5.0) | 0.60 |

Antibiotic 19A has a characteristic and unique ultraviolet spectrum. Figure 1 shows the spectrum in 0.1 N hydrochloric acid, in methanol, and in 0.1 M phosphate buffer (pH 7.0). Measurements were taken with a Cary recording spectrophotometer.

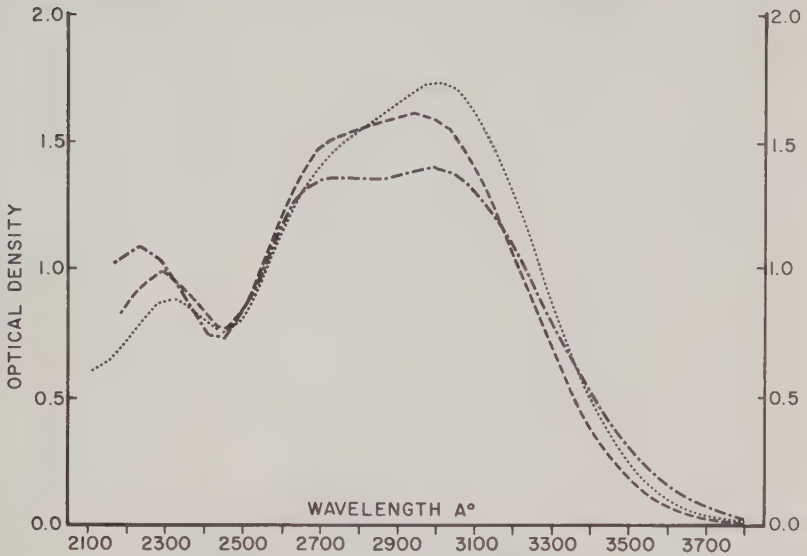



FIG. 1. *Ultraviolet absorption spectra of antibiotic 19A.* ——— *MeOH;* —.—.—.— *0.1 M phosphate (pH 7.0);* .....*0.1 N HCl.*

In 0.1 N HCl $E^{1\%}_{1\ cm}$ = 882 at 3030 A, max; $E^{1\%}_{1\ cm}$ = 740 at 2790 A, inf; $E^{1\%}_{1\ cm}$ = 433 at 2330 A, max. In 0.1 M pH 7.0 phosphate buffer $E^{1\%}_{1\ cm}$ = 697 at 3010 A, max; $E^{1\%}_{1\ cm}$ = 658 at 2730 A, max; $E^{1\%}_{1\ cm}$ = 517 at 2270 A, max. In methanol $E^{1\%}_{1\ cm}$ = 777 at 2950 A, max; $E^{1\%}_{1\ cm}$ = 742 at 2800 A, inf; $E^{1\%}_{1\ cm}$ = 486 at 2230 A, max.

The infrared spectrum of antibiotic 19A obtained by use of a potassium bromide pellet, is shown in Fig. 2. A Baird model B spectrophotometer was used.

The antibiotic is insoluble in water, benzene, chlorinated solvents, petroleum ether, and dilute aqueous acids at ambient temperatures. Antibiotic 19A is partially soluble in methanol, ethanol, butanol, acetone, methyl isobutyl ketone, ethyl acetate, and amyl acetate. The antibiotic is soluble in pyridine, glacial acetic acid, dioxane, formamide, aqueous alkali, aqueous sodium carbonate, and bicarbonate solutions.

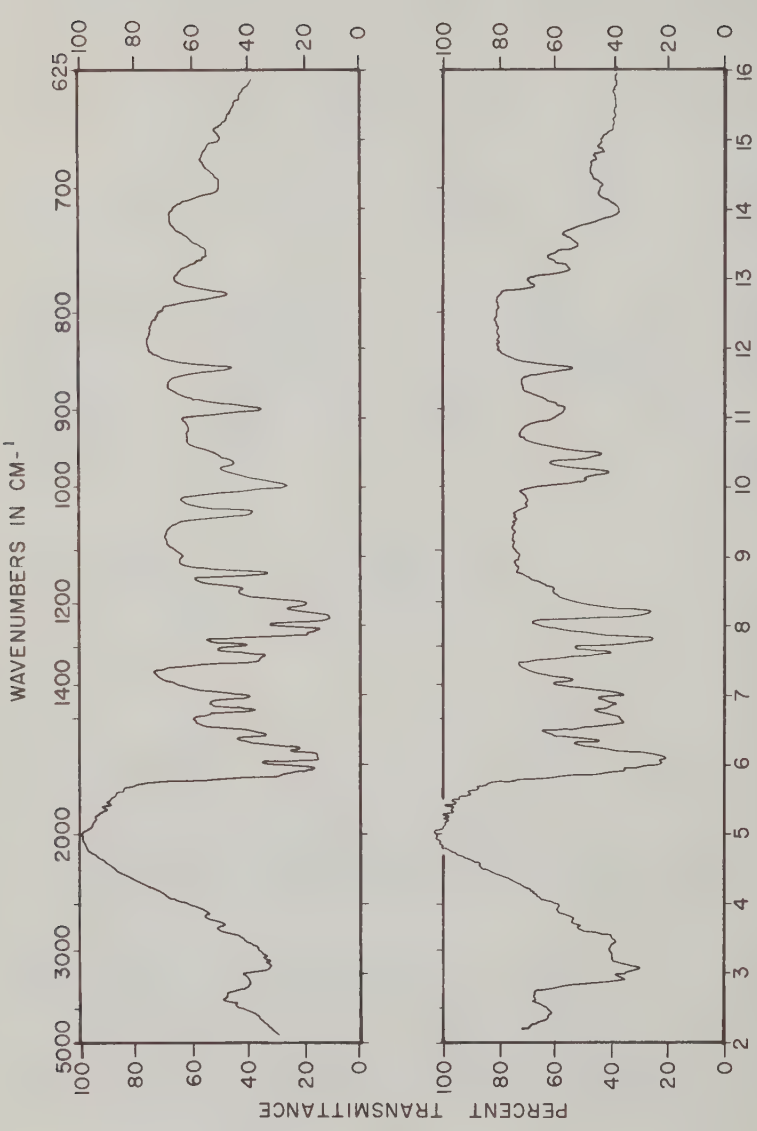



FIG. 2. *(top) Infrared absorption spectrum of antibiotic 19A. (bottom) Spectrum of the ammonia degradation product.*

Crystalline antibiotic 19A gives negative Molisch, biuret, Somogyi, Millon, ninhydrin, Sakaguchi, Benedict, and Fehling tests. It gives a positive Tollen test.

Antibiotic 19A can be crystallized as well-defined white needles, which are birefringent under polarized light. A powdered X-ray diffraction pattern showed the material to be crystalline. A melting point of 162 to 166 C (decomp.) was obtained in an open capillary tube. A phase purity analysis indicated a purity of 98.0 ± 0.5% for the crystalline acid. Water at 50 C was used as the solvent. An extrapolated solubility of 0.70 mg/ml was obtained.

Titration of antibiotic 19A with lithium hydroxide in 50% methanol-water indicated an equivalent weight of 171, with a $pH_{\frac{1}{2}}$ of 4.5.

Table 5 lists the elemental analysis data for antibiotic 19A. The elemental analysis indicates a molecular formula corresponding to $C_{11}H_{13}N_4O_9$ and a molecular weight of 345.

TABLE 5. *Elemental analysis of antibiotic 19A*

| Element | Found | Calculated |
|---|---|---|
| C | 38.3 | 38.3 |
| H | 3.79 | 3.77 |
| N | 16.2 | 16.2 |
| O | 41.7* | 41.7* |

*By difference.

On standing in 0.1 N sodium hydroxide, the ultraviolet absorption maximum in the 3000 A region rapidly disappears, with the concurrent appearance of a new peak (Fig. 3).

In 0.1 N NaOH $E^{1\%}_{1\ cm}$ = 351 at 2970 A, inf; $E^{1\%}_{1\ cm}$ = 807 at 2600 A, max. The ultraviolet absorption spectrum, characteristic of antibiotic 19A, is not obtained upon reacidification of the alkali-treated sample. These spectra could be used as a chemical assay method for measuring antibiotic yields in the isolation process.

Crystalline 19A on standing in ammonia overnight yields, upon evaporation, a product having no antibacterial activity.

The ultraviolet spectra in water, acid, and alkali are shown in Fig. 4.

In $H_2O$ $E^{1\%}_{1\ cm}$ = 745 at 2850 A, max; $E^{1\%}_{1\ cm}$ = 692 at 2690 A, inf; $E^{1\%}_{1\ cm}$ = 459 at 2180 A, max. In 0.1 N HCl $E^{1\%}_{1\ cm}$ = 667 at 2775 A, max; $E^{1\%}_{1\ cm}$ = 652 at 2670 A, inf. In 0.1 N NaOH $E^{1\%}_{1\ cm}$ = 390 at 3050 A, max; $E^{1\%}_{1\ cm}$ = 586 at 2550 A, max.

The infrared spectrum, obtained by use of a potassium bromide pellet, of the ammonia degradation product is shown in Fig. 2. This product has a melting point >300 C and an $R_F$ = 0.0 in a phosphate (pH 5.0) buffer-methyl isobutyl ketone system.

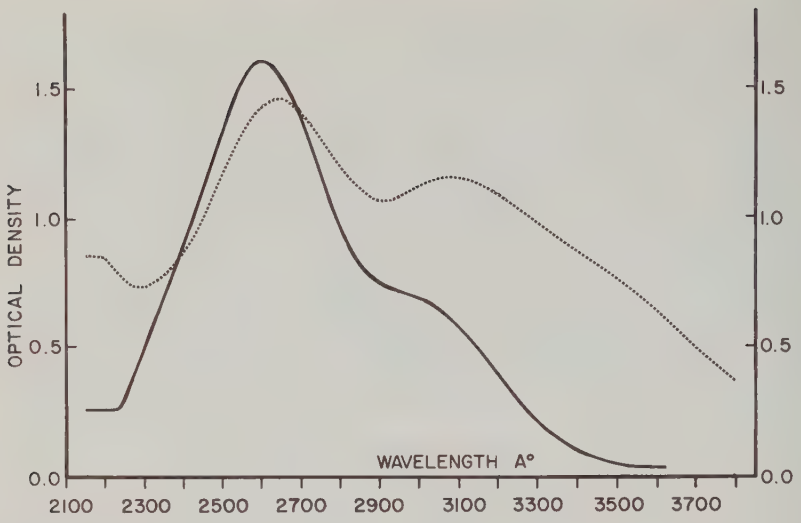



FIG. 3. *Ultraviolet absorption spectra of the alkali degradation product of antibiotic 19A.* ———— *0.1 N NaOH, 2 hr;* ..... *0.1 N NaOH, 10 min.*

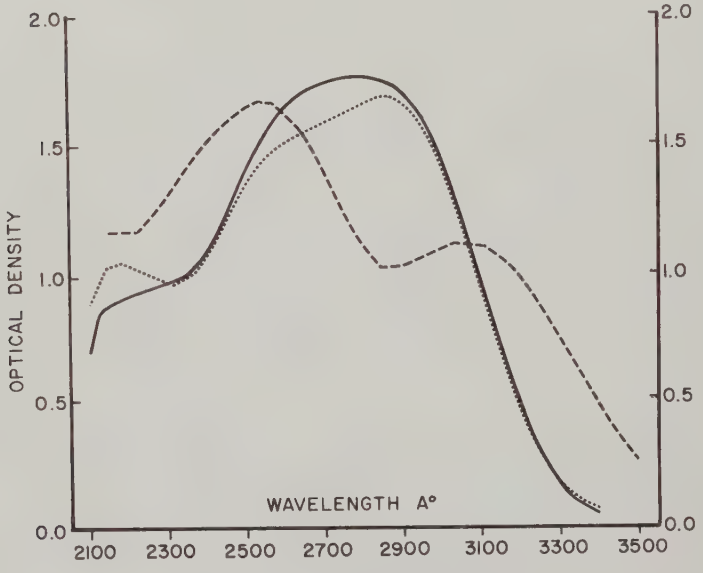



FIG. 4. *Ultraviolet absorption spectra of the ammonia degradation product of antibiotic 10A.* ..... $H_2O$; ———— *0.1 N HCl;* – – – – – – *0.1 N NaOH.*

On hydrogenation with palladium on charcoal, antibiotic 19A absorbed 7 moles of hydrogen. The resulting product had no antibiotic activity.

## Literature Cited

Miller, B. M., A. Barreto, Jr., and H. B. Woodruff. 1962. A crystalline antibiotic from a thermophilic streptomycete. I. Biological properties. Antimicrobial Agents and Chemotherapy - 1961, p. 445-453.

# BIOLOGICAL STUDIES OF 6-METHYLENE OXYTETRACYCLINE, A NEW TETRACYCLINE

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With the modification of the various substituent groups along the "upper periphery" of the basic tetracycline molecule, several new tetracycline antibiotics have been discovered (Stephens et al., 1958; Berreboom et al., 1960; Blackwood et al., 1961). The biological properties of one of these, 6-methylene oxytetracycline, indicate the possibility of unusual effectiveness as a therapeutic agent against diverse bacterial infections.

## Materials and Methods

6-Methylene oxytetracycline samples were supplied to this laboratory by members of the Chemical Research Division of Chas. Pfizer and Co., Inc. In each instance, the preparation was of research purity and used as 100% pure material. In all studies, 6-methylene oxytetracycline was compared with research-grade samples of commercially-available 6-demethylchlortetracycline (McCormick et al., 1957; Kunin and Finland, 1958). In some instances, tetracycline was included also for comparative purposes.

The microorganisms were maintained as stock cultures in this laboratory by daily transfer in brain heart infusion broth (Difco). All the reported in vitro studies were done in this medium. Incubation was at 37 C for 18 to 20 hr.

In the chemotherapeutic studies, white mice were infected by the intraperitoneal route. The infecting organism used was administered in 5% hog gastric mucin. Therapy was initiated 0.5 hr after infection; single dosage was used by either the oral or subcutaneous route. These methods have been described in detail in a number of previous publications from this laboratory (English et al., 1952; English and McBride, 1959).

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## Results and Discussion

Minimal inhibitory concentrations (MIC), as determined by an arithmetic progression-dilution technique against eight common pathogenic bacteria, are presented in Table 1 (Tompsett, Shultz, and McDermott, 1947). In each instance the standard deviation is presented (N = 6). 6-Methylene oxytetracycline demonstrated greater in vitro activity against *Streptococcus pyogenes* 98 and *S. faecalis* than did 6-demethylchlortetracycline. The remaining six bacteria show the same MIC's, within the calculated standard deviation, for both 6-methylene oxytetracycline and 6-demethylchlortetracycline.

TABLE 1. *In vitro activity of 6-methylene oxytetracycline against representative gram-positive bacteria with 6-demethychlortetracycline and tetracycline included for comparative purposes*

| Organism | MIC* with standard deviation | | |
|---|---|---|---|
| | 6-Methylene oxytetracycline | 6-Demethylchlor-tetracycline | Tetracycline |
| | *μg/ml* | *μg/ml* | *μg/ml* |
| *Staphylococcus aureus* 5 | 0.220 ± 0.076 | 0.260 ± 0.048 | 0.360 ± 0.048 |
| *S. aureus* 209P | 0.160 ± 0.048 | 0.180 ± 0.040 | 0.320 ± 0.066 |
| *Streptococcus pyogenes* | 0.032 ± 0.004 | 0.032 ± 0.004 | 0.064 ± 0.008 |
| *S. pyogenes* 98 | 0.040 ± 0.001 | 0.048 ± 0.003 | 0.088 ± 0.009 |
| *Diplococcus pneumoniae* | 0.220 ± 0.040 | 0.160 ± 0.048 | 0.260 ± 0.048 |
| *Streptococcus faecalis* | 0.140 ± 0.048 | 0.240 ± 0.048 | 0.260 ± 0.048 |
| *Escherichia coli* | 0.740 ± 0.048 | 0.740 ± 0.048 | 0.760 ± 0.048 |
| *Klebsiella pneumoniae* | 0.300 ± 0.020 | 0.300 ± 0.020 | 0.460 ± 0.078 |

*Minimal inhibitory concentration (MIC) refers to the least amount of antibiotic that prevented visible growth of the test organism within 18 to 20 hr of incubation at 37 C. Inoculum: 0.5 ml of $1 \times 10^{-3}$ dilution of 18- to 20-hr test culture. Medium: brain heart infusion broth (Difco).

The in vitro activities of 6-methylene oxytetracycline, 6-demethylchlortetracycline, and tetracycline against 21 other representative, pathogenic bacteria and *Candida albicans*, as determined by the common twofold, serial-dilution technique, are presented in Table 2. These are representative average values taken from a number of different experiments done on different days. 6-Methylene oxytetracycline and 6-demethylchloretetracycline, as determined in this technique, have similar MIC's against the gram-positive bacteria. In most instances, tetracycline was less active by a single tube in the dilution series. Against gram-negative bacteria, activity differences between these three antibiotics usually represented one tube in the dilution series. None of these antibiotics had activity against *C. albicans*. *Staphylococcus aureus* strains 376 and 400 are resistant to oxytetracycline, chlortetracycline, and tetracycline in addition to a number of the other commercial antibiotics. It is noted that 6-methylene oxytetracycline shows apparent cross-reference between these established

tetracyclines. Similar results were obtained here with 6-demethylchlortetracycline and tetracycline against these antibiotic-resistant staphylococci.

TABLE 2. *In vitro spectrum of 6-methylene oxytetracycline with 6-demethylchlortetracycline and tetracycline included for comparative purposes*

| Organism | MIC* | | |
|---|---|---|---|
| | 6-Methylene oxytetracycline | 6-Demethylchlor-tetracycline | Tetracycline |
| | *μg/ml* | *μg/ml* | *μg/ml* |
| *Streptococcus pyogenes* | 0.019 | 0.015 | 0.039 |
| *Erysipelothrix insidiosa* | 0.078 | 0.125 | 0.390 |
| *Corynebacterium diphtheriae* | 0.312 | 0.500 | 0.780 |
| *Listeria monocytogenes* | 0.156 | 0.190 | 0.250 |
| *Bacillus subtilis* | 0.031 | 0.031 | 0.078 |
| *Streptococcus agalactiae* | 0.125 | 0.125 | 0.250 |
| *Aerobacter aerogenes* | 0.39 | 0.78 | 0.78 |
| *Escherichia coli* | 0.312 | 0.312 | 0.490 |
| *Salmonella typhosa* | 0.390 | 0.780 | 0.655 |
| *Salmonella gallinarum* | 1.56 | 1.25 | 1.40 |
| *Neisseria gonorrheae* | 0.312 | 0.19 | 0.312 |
| *Bordetella pertussis* | 0.09 | 0.19 | 0.19 |
| *Shigella sonnei* | 0.39 | 0.78 | 0.78 |
| *Bordetella bronchiseptica* | 0.09 | 0.09 | 0.26 |
| *Actinobacillus mallei* | 0.39 | 0.39 | 0.78 |
| *Vibrio comma* | 0.39 | 0.19 | 0.78 |
| *Pasteurella multocida* | 0.78 | 0.39 | 0.78 |
| *Proteus vulgaris* | >100 | >100 | >100 |
| *Pseudomonas aeruginosa* | >100 | >100 | >100 |
| *Candida albicans* | >100 | >100 | >100 |
| *Staphylococcus aureus* (antibiotic-resistant)† | | | |
| St376 | >100 | >100 | >100 |
| St400 | >100 | >100 | >100 |

*See footnote to Table 1.

†Resistant to high concentrations of oxytetracycline, chlortetracycline, tetracycline, and other commercial antibiotics.

Figures 1 and 2 give summaries of the relative activity rankings of 6-methylene oxytetracycline, 6-demethylchlortetracycline, and tetracycline for 189 gram-positive and 225 gram-negative bacteria. All were clinical isolates from hospitals in widely separated geographical areas. The individual data, obtained by hospital personnel, were ranked as follows: the most active antibiotic (i.e., lowest MIC) against a test organism was assigned a value of 3; the next most active antibiotic was assigned a value of 2; and the least active antibiotic was assigned 1. Total scoring for each antibiotic was divided by the number of times it was studied so that all antibiotics were assessed on an equal basis. Of course, only those considered susceptible

to the antibiotics were included in this comparison. The arbitrary "susceptibility" range for gram-positive bacteria was MIC values less than 6 μg/ml; the cut-off point for gram-negative bacteria was MIC values less than 12 μg/ml.

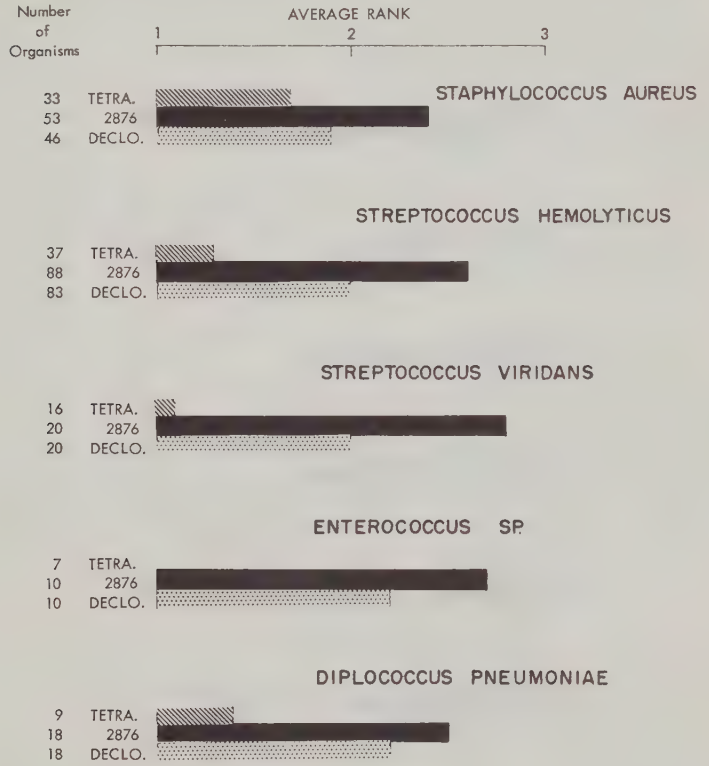


FIG. 1. *Relative activity rankings of gram-positive organisms. Symbols:* Tetra = *tetracycline;* 2876 = *6-methylene oxytetracycline; and* Declo = *6-demethylchlortetracycline.*

6-Methylene oxytetracycline was ranked as the most active antibiotic against the 189 cultures representing 7 genera of gram-positive bacteria (Fig. 1). 6-Demethylchlortetracycline was ranked second in activity and tetracycline, third. Differences between 6-methylene oxytetracycline and 6-demethylchlortetracycline were most evident against various hemolytic and Viridans streptococcal strains. 6-Methylene oxytetracycline was most active against the six *Proteus* and 51 *Pseudomonas* strains studied (Fig. 2). 6-Methylene oxytetracycline and 6-demethylchlortetracycline demonstrated equal activity

against two strains of *Shigella*. 6-Demethylchlortetracycline was most active against *Escherichia coli*, *Klebsiella pneumoniae*, paracolon bacteria, and *Salmonella* species. Tetracycline was less active than the other two antibiotics in each instance.

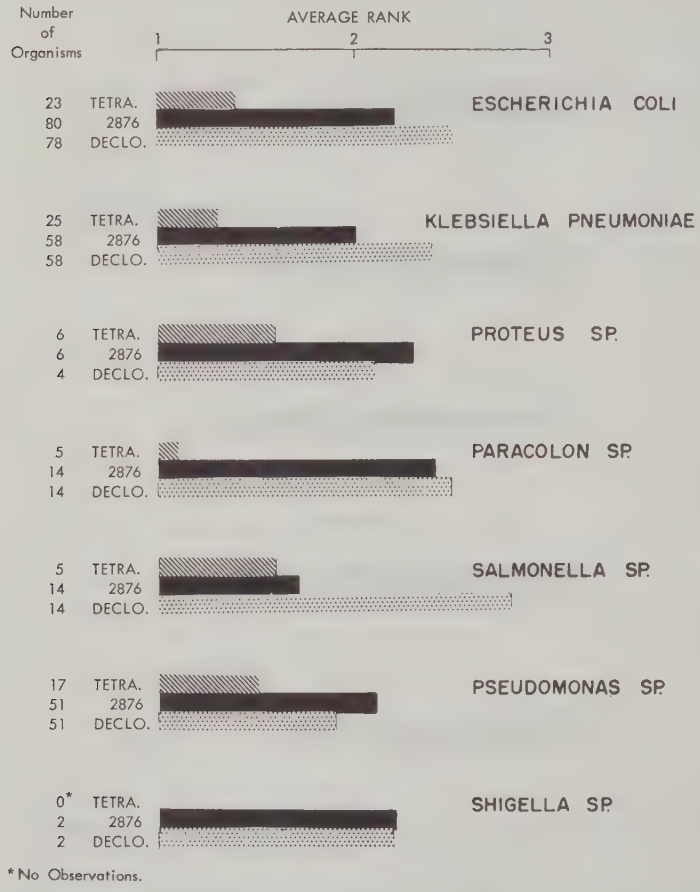


FIG. 2. *Relative activity rankings of gram-negative organisms. Symbols: see Fig. 1.*

*S. aureus* and *E. coli*, when transferred in gradually increasing concentrations of 6-methylene oxytetracycline, showed only a gradual, step-wise increase in resistance to the drug. After 15 transfers (Table 3), an eightfold increase was observed for 6-methylene oxytetracycline resistance. In the same experiment, 6-demethylchlortetracycline showed a 16-fold increase. *E. coli*, in the same

experiment, showed a final fourfold increase for both 6-methylene oxytetracycline and 6-demethylchlortetracycline. Similar results, i.e., a gradual, step-wise increase in contrast to a one-step acquisition of resistance, have been obtained previously with other tetracycline antibiotics (English and Gelwicks, 1951; English et al., 1954).

TABLE 3. *In vitro resistance pattern of Staphylococcus aureus and Escherichia coli to 6-methylene oxytetracycline with 6-demethylchlortetracycline included for comparative purposes*

| Organism | Antibiotic | Initial MIC | Number of transfers | Final MIC | Fold increase in resistance |
|---|---|---|---|---|---|
| *Staphylococcus aureus* | 6-Methylene oxytetracycline | 0.19 | 15 | 1.56 | 8 |
| | 6-Demethylchlor-tetracycline | 0.19 | 15 | 3.12 | 16 |
| *Escherichia coli* | 6-Methylene oxytetracycline | 0.39 | 15 | 1.56 | 4 |
| | 6-Demethylchlor-tetracycline | 0.39 | 15 | 1.56 | 4 |

The results of antibiotic-susceptibility testing, using freshly prepared discs containing 5 and 30 $\mu$g of 6-methylene oxytetracycline or 6-demethylchlortetracycline against a variety of common bacteria, are presented in Table 4. Of the eight bacteria tested with 5-$\mu$g discs, 6-methylene oxytetracycline produced larger zone sizes against four bacteria than did 6-demethylchlortetracycline. With the 30/$\mu$g disc,

TABLE 4. *Zone sizes produced by 6-methylene oxytetracycline against susceptible bacteria with 6-demethylchlortetracycline included for comparative purposes*

| Organism | Average zone sizes* | | | |
|---|---|---|---|---|
| | 6-Methylene oxytetracycline | | 6-Demethylchlor-tetracycline | |
| | 5 $\mu$g disc | 30 $\mu$g disc | 5 $\mu$g disc | 30 $\mu$g disc |
| | *mm* | *mm* | *mm* | *mm* |
| *Staphylococcus aureus* | 27.2 | 34.5 | 26.4 | 30.1 |
| *Streptococcus faecalis* | 25.7 | 31.9 | 27.0 | 32.6 |
| *Streptococcus pyogenes* | 33.0 | 35.0 | 34.0 | 35.0 |
| *Diplococcus pneumoniae* | 27.1 | 33.2 | 25.5 | 31.4 |
| *Erysipelothrix insidiosa* | 26.7 | 34.0 | 25.4 | 35.0 |
| *Corynebacterium diphtheriae* | 15.6 | 23.3 | 16.4 | 21.2 |
| *Bacillus subtilis* | 32.3 | 35.0 | 30.6 | 34.3 |
| *Listeria monocytogenes* | 28.7 | 35.0 | 29.2 | 34.3 |
| *Staphylococcus aureus* (antibiotic-resistant) St400 | 0 | 0 | 0 | 0 |

*Average of three different tests. If zone size was >35, it was averaged as 35.

6-methylene oxytetracycline produced larger zones against four bacteria; 6-demethylchlortetracycline produced larger zones against two; and equal zones were produced by these antibiotics against two bacteria.

It was obvious at this stage in the evaluation, that differences in in vitro activities between 6-methylene oxytetracycline and 6-demethylchlortetracycline were not striking. Therefore, data obtained in concomitant in vivo experiments against experimental *S. aureus* infection in mice, which indicated a pronounced superiority for 6-methylene oxytetracycline, were unexpected. It was decided, therefore, that extensive chemotherapeutic evaluation of 6-methylene oxytetracycline against a series of experimental infections in mice was warranted (Table 5). Again, in each instance, 6-demethylchlortetracycline is included for comparative purposes. Data are presented in terms of $PD_{50}$, i.e., the amount of antibiotic that protects 50% of the animals. These calculations follow the method of Reed and Muench (1938).

TABLE 5. *Chemotherapeutic efficacy of 6-methylene oxytetracycline against experimental intraperitoneal infections in mice with 6-demethylchlortetracycline included for comparative purposes*

| Infection | $PD_{50}$ in mg/kg with standard deviation* | | | |
|---|---|---|---|---|
| | 6-Methylene oxytetracycline | | 6-Demethylchlortetracycline | |
| | Oral | Subcutaneous | Oral | Subcutaneous |
| *Staphylococcus aureus* | 1.50 (1.87 - 1.20)† | 0.28 (0.34 - 0.22) | 4.6 (5.75 - 3.68) | 0.95 (1.19 - 0.76) |
| *Escherichia coli* | 53 (±6.3) | 6.5 (±0.11) | 140 (±5) | 20 (±1.6) |
| *Streptococcus pyogenes* | 8.33 (±2.33) | 0.64 (±0‡) | 12.6 (±0.99) | 0.99 (±0.084) |
| *Aerobacter aerogenes* | 89.5 (±9.1) | 7.2 (±0.5) | 84.5 (±3.6) | 5.35 (±0.8) |
| *Diplococcus pneumoniae* | 45 (±2.7) | 11.1 (±0.8) | 87.3 (±11) | 14.5 (±1.1) |
| *Klebsiella pneumoniae* | 110 (±1.4) | 17.1 (±0.9) | >200 | >40 |
| *Salmonella typhosa* | 37 (±1.4) | 9.88 (±0.22) | 47.8 (±3) | 11.04 (±1.5) |

*Protective dose 50% ($PD_{50}$) is that amount of drug which protected 50% of the animals. (Calculated by the Reed and Muench method.) Therapy was initiated 0.5 hr postinfection with single dosage.

†This value and confidence limits were obtained by the method of Litchfield and Wilcoxon (1949).

‡Same value in all tests.

The in vivo chemotherapeutic efficacy of 6-methylene oxytetracycline over 6-demethylchlortetracycline is most striking against the experimental *S. aureus* and *E. coli* infections. 6-Methylene oxytetracycline, administered either orally or subcutaneously, was

approximately 200 to 300% more active against these infections than was 6-demethylchlortetracycline. Against an experimental *S. pyogenes* infection, 6-methylene oxytetracycline was approximately 20 to 40% more active than was 6-demethylchlortetracycline; against an experimental *Diplococcus pneumoniae* infection, it was 20 to 60% more active.

6-Methylene oxytetracycline produced an oral $PD_{50}$ of 110 (±1.4 mg/kg) and a parenteral $PD_{50}$ of 17.1 (±0.9 mg/kg) against our experimental *K. pneumoniae* infection in mice. In contrast, the oral $PD_{50}$ for 6-demethylchlortetracycline was >200 mg/kg, and the parenteral, >40 mg/kg against this same infection. In an earlier study, chlortetracycline had shown a similar poor protective effect against this particular infection (English et al., 1954). Against the experimental *Salmonella typhosa* infection in mice, 6-methylene oxytetracycline was approximately 20% more active than 6-demethylchlortetracycline after oral administration. However, when administered by the parenteral route, both 6-methylene oxytetracycline and 6-demethylchlortetracycline produced similar $PD_{50}$ values. The $PD_{50}$ values obtained by either the oral or subcutaneous routes of administration of both antibiotics against the experimental *Aerobacter aerogenes* infection were, in effect, equivalent.

TABLE 6. *Serum activity in beagle dogs after oral administration of 6-methylene oxytetracycline with 6-demethylchlortetracycline included for comparative purposes*

| Antibiotic | Oral dosage | Avg serum activity (time in hr)* | | | | |
|---|---|---|---|---|---|---|
| | | 0 | 1 | 3 | 5 | 7 |
| | *mg/kg* | | | | | |
| 6-Methylene oxytetracycline | 10 | 0 | 14 | 22 | 18 | 10 |
| 6-Demethylchlortetracycline | 10 | 0 | 5.5 | 6 | 5 | 2 |
| 6-Methylene oxytetracycline | 20 | 0 | 48 | 128 | 64 | 64 |
| 6-Demethylchlortetracycline | 20 | 0 | 13 | 16 | 16 | 10 |

*Activity is expressed as an average of dilution reciprocals. The organism, *S. pyogenes*, was assayed by twofold serial dilution technique.

6-Methylene oxytetracycline produced consistently higher serum antibacterial activity after oral administration to beagle dogs than did 6-demethylchlortetracycline (Table 6). After oral dosage of 10 mg/kg, 6-methylene oxytetracycline activity in the serum, measured against a pathogenic *Streptococcus*, was two to four times higher than the corresponding activity shown by the same dosage of 6-demethylchlortetracycline. After oral dosage of 20 mg/kg, these differences became even more pronounced. Here, serum activity of 6-methylene oxytetracycline ranged from four to eight times higher

than that achieved with the same dosage of 6-demethylchlortetracycline.

Preliminary studies in the beagle dog on urinary excretion of the total administered dose demonstrated that 6-methylene oxytetracycline as compared with 6-demethylchlortetracycline had a slower rate of excretion, as well as the fact that lower proportions of the administered dose were detected in the urine. Perhaps this offers an explanation for the high serum levels of 6-methylene oxytetracycline.

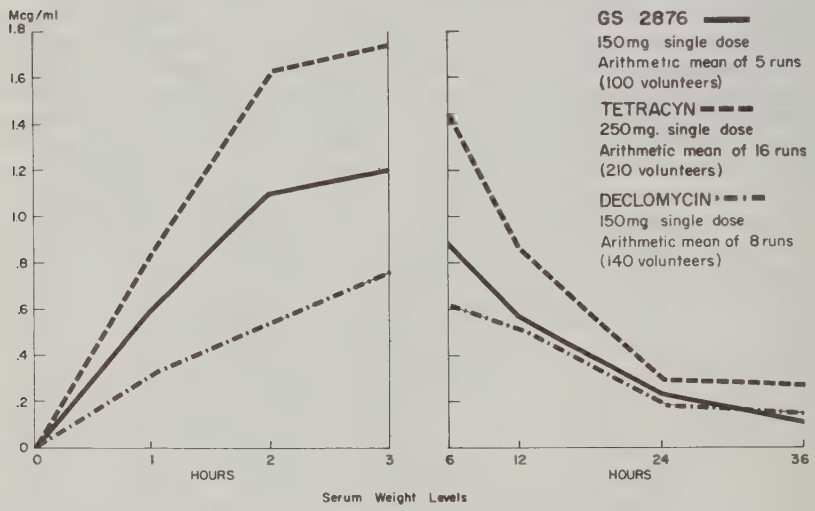


FIG. 3. *Serum concentrations (μg/ml) after oral administration to human volunteers. Antibiotics were assayed in terms of their own standards. Symbols:* GS 2876 = *6-methylene oxytetracycline (150 mg; single dose; arithmetic mean of five runs with 100 volunteers);* Tetracyn = *tetracycline (250 mg; single dose; arithmetic mean of 16 runs with 210 volunteers); and* Declomycin = *6-demethylchlortetracycline (150 mg; single dose; arithmetic mean of eight runs with 140 volunteers).*

In Fig. 3, serum levels (expressed in μg/ml) of 6-methylene oxytetracycline and 6-demethylchlortetracycline, after oral dosage of 150 mg to normal volunteers, are compared. The data for 6-methylene oxytetracycline are arithmetic means of five separate experiments which included 100 volunteers. The data for 6-demethylchlortetracycline are arithmetic means of eight separate experiments which included 140 volunteers. Thus, sera were assayed in terms of their own standard by the common microbiological procedure (Grove and Randall, 1955). It is obvious that 6-methylene oxytetracycline produced higher serum concentrations (μg/ml) than did 6-demethylchlortetracycline. During the first 6 hr, the concentration of 6-methylene

oxytetracycline is approximately double that of 6-demethylchlortetracycline. The 12-, 24-, and 36-hr serum concentrations of these antibiotics gradually became equivalent. It is noteworthy that activity was still detected 36 hr after single dosage of 150 mg. Data for tetracycline, administered orally at an accepted dosage in man of 250 mg, are also included in Fig. 3. It would appear that this oral absorption of 6-methylene oxytetracycline and tetracycline must be quite similar. This dosage of tetracycline is 60% higher than that studied with 6-methylene oxytetracycline. During the 1- to 62-hr period, serum concentration of tetracycline in μg/ml was an average of 50% (30 to 70% range) higher than 6-methylene oxytetracycline. Therefore, serum concentrations are considerably higher for tetracycline than are those obtained with 6-demethylchlortetracycline.

Serum activity, expressed as geometric means of dilution reciprocals, for 6-methylene oxytetracycline and 6-demethylchlortetracycline are presented in Table 7, which summarizes a three-way crossover study. Each figure represents a total of 30 volunteers. The usual twofold, serial dilution technique was used with four different assay

TABLE 7. *Serum activity of 6-methylene oxytetracycline, 6-demethylchlortetracycline, and tetracycline after oral administration to normal volunteers*

| Antibiotic* | Assay organism | Antibacterial activity in the serum† | | | | | |
|---|---|---|---|---|---|---|---|
| | | 1 hr | 3 hr | 6 hr | 12 hr | 24 hr | 36 hr |
| 6-Methylene oxytetracycline | *Streptococcus* 98 | 3.66 | 11.2 | 6.36 | 2.51 | 0 | 0 |
| 6-Demethylchlortetracycline | *Streptococcus* 98 | 1.58 | 7.11 | 7.62 | 2.73 | 0 | 0 |
| Tetracycline | *Streptococcus* 98 | 5.24 | 14.9 | 14.5 | 4.2 | 1.07 | 0 |
| 6-Methylene oxytetracycline | *Streptococcus* 9282 | 3.25 | 13.22 | 8.80 | 3.03 | 0 | 0 |
| 6-Demethylchlortetracycline | *Streptococcus* 9282 | 1.91 | 8.38 | 8.60 | 2.93 | 0 | 0 |
| Tetracycline | *Streptococcus* 9282 | 5.38 | 18.5 | 16.8 | 5.17 | 1.23 | 0 |
| 6-Methylene oxytetracycline | *Diplococcus* I | 6.64 | 26.5 | 20.1 | 8.57 | 0 | 0 |
| 6-Demethylchlortetracycline | *Diplococcus* I | 3.48 | 16 | 13.6 | 8 | 0 | 0 |
| Tetracycline | *Diplococcus* I | 8.82 | 25.8 | 24.9 | 8.82 | 1.27 | 1.07 |
| 6-Methylene-oxytetracycline | *Staphylococcus* DB 13 | 1.29 | 3.25 | 2.25 | 1.05 | 0 | 0 |
| 6-Demethylchlortetracycline | *Staphylococcus* DB 13 | 0 | 1.45 | 1.55 | 1.12 | 0 | 0 |
| Tetracycline | *Staphylococcus* DB 13 | 1.23 | 3.27 | 2.73 | 1.10 | 1.05 | 0 |

*Dosage: 6-methylene oxytetracycline, 300 mg, single; 6-demethylchlortetracycline, 300 mg, single; and tetracycline, 500 mg, single.

†Results are expressed as the geometric mean of the dilution reciprocals. All 0-hr samples were negative in the three-way, crossover study. Each value represents 30 volunteers.

microorganisms. As the data are presented, 6-methylene oxytetracycline produced greater activity than did 6-demethylchlortetracycline at nearly every time sample from 1 through 6 hr. Higher geometric means at 12 hr were also found with 6-methylene oxytetracycline after assay with *Streptococcus* 9282 and *Diplococcus* 1 than for 6-demethylchlortetracycline. The converse of this is true after assay with *Streptococcus* 98 and *Staphylococcus* DB13. Serum activity was not detected at 24 or 36 hr after dosage with either 6-methylene oxytetracycline or 6-demethylchlortetracycline. Serum activity of tetracycline after oral dosage of 500 mg is also included in Table 7 for comparative purposes. In nearly every instance, the geometric mean as presented is higher than that for 6-methylene oxytetracycline and 6-demethylchlortetracycline at the same time. However, statistical analysis shows a significant difference ($P = 0.05$) between the geometric means of tetracycline and 6-methylene oxytetracycline only in the 6-hr value in the assay using *Streptococcus* 98. In direct contrast, all values reported for 6-demethylchlortetracycline at 1, 3, and 6 hr, with each organism, are significantly lower than the values attained with tetracycline. (We are indebted to D. Hucke, statistician of the Chas. Pfizer and Co., Inc., Brooklyn, N.Y., for this statistical analysis.)

## Literature Cited

Beereboom, J. J., J. J. Ursprung, H. H. Rennhard, and C. R. Stephens. 1960. Further 6-deoxytetracycline studies: effect of aromatic substituents on biological activity. J. Am. Chem. Soc. **82**:1003.

Blackwood, R. K., J. J. Beereboom, H. H. Rennhard, M. Schach von Wittenau, and C. R. Stephens. 1961. 6-Methylene tetracyclines: a new class of tetracycline antibiotics.. J. Am. Chem. Soc. **83**:2773.

English, A. R., M. F. Field, S. R. Szendy, N. J. Tagliani, and R. A. Fitts. 1952. Magnamycin. I. In vitro studies. Antibiotics and Chemotherapy **2**:678-688.

English, A. R., and P. C. Gelwicks. 1951. The in vitro microbial resistance pattern to Terramycin. In vitro studies. Antibiotics & Chemotherapy **1**:118-124.

English, A. R., and T. J. McBride. 1959. Laboratory evaluations of partially acetylated esters of oleandomycin. Proc. Soc. Exptl. Biol. Med. **100**:880-884.

English, A. R., S. Y. P'an, T. J. McBride, J. F. Gardocki, G. Van Halsema, and W. A. Wright. Tetracycline - microbiologic, pharamcologic, and clinical evaluation. Antibiotics Ann. 1953-1954, p. 70-80.

Grove, D. C., and W. A. Randall. 1955. Assay methods of antibiotics, a laboratory manual. Medical Encyclopedia, Inc., New York.

Kunin, C. M., and M. Finland. 1958. Demethylchlortetracycline - A new tetracycline antibiotic that yields greater and more sustained antibacterial activity. New Engl. J. Med. **259**: 999-1005.

Litchfield, J. T., and F. Wilcoxan. 1949. A simplified method of evaluating dose-effect experiments. J. Pharmacol. Exptl. Therap. **96**:99-113.

McCormick, J. R. D., N. O. Sjolander, U. Hirsch, E. R. Jensen, and A. P. Doerschuk. 1957. New family of antibiotics: demethyltetracyclines. J. Am. Chem. Soc. **78**:4561-4563.

Reed, L. J., and H. Muench. 1938. A simple method of estimating fifty per cent end points. Am. J. Hyg. **27**:493-497.

Stephens, C. R., K. Murai, H. H. Rennhard, L. H. Conover, and K. Brunings. 1958. Hydrogenolysis studies in the tetracycline series - 6 Deoxytetracyclines. J. Am. Chem. Soc. 80:5324.

Tompsett, R., S. Shultz, and W. McDermott. 1947. The relation of protein binding to the pharmacology and antibacterial activity of penicillins X, G, dihydro F, and K. J. Bacteriol. 53: 581-595.

# CRYSTALLINE ANTIFUNGAL ANTIBIOTIC ISOLATED FROM A STRAIN OF *STREPTOMYCES RUTGERSENSIS*

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A new antifungal antibiotic, A272, has been obtained from a streptomycete isolated from soil. The general paper chromatographic behavior of the antifungal activity, as found in broth, resembled that of the oligomycins. However, A272 could be separated from the oligomycins in certain solvent systems. Since it appeared to be an unidentified antifungal antibiotic, work was initiated to obtain the substance in crystalline form.

This communication will describe the organism and medium employed for production of A272, the isolation procedure, and some of the physical, chemical, and biological properties of the crystalline antifungal compound.

## Materials and Methods

*Organism.* The A272-producing organism appeared similar to *Streptomyces arabicus* NRRL 1733, *S. parvullus* NRRL 1628, and *S. rutgersensis* NRRL B-1256. A cultural comparison of these organisms on different media indicated that the A272-producing organism could be regarded as a strain of *S. rutgersensis* NRRL B-1256, although certain differences did exist.

These two cultures were similar in the following characteristics: spore chain morphology, spore morphology, spore color, color of vegetative mycelia on most media, absence of $H_2S$ production, absence of nitrate reduction, and utilization of 16 carbon sources. However, the A272-producing culture differed in that it did not coagulate milk, did not liquefy gelatin, grew differently on salts-starch agar, did not utilize D-sorbitol, and utilized L(+)-rhamnose.

*S. rutgersensis* was the only organism of the three listed which produced an antifungal antibiotic. A mixture of polyene-antifungal antibiotics was extracted from the broth and mycelia of *S. rutgersensis*. Ultraviolet-absorption studies indicated that this polyene mixture

contained both a pentaene and a hexaene compound. Paper chromatographic data showed that *S. rutgersensis* did not produce A272.

*Fermentation of A272.* A272 was produced in 10-gallon stainless steel fermentors in a medium which contained the following ingredients in g/liter $H_2O$: glucose, 10; Brer Rabbit molasses, 20; Bacto peptone, 5; and $CaCO_3$, 2, pH 7.1. This fermentation was incubated at 30 C for 59 hr. Another medium, which proved satisfactory for the production of A272, contained the following ingredients in g/liter $H_2O$: casein, 5; dextrose, 30; blackstrap molasses, 10; yeast 2019, 5; and $CaCO_3$, 5, pH 6.2.

*Microbiological assay of A272.* A272 was assayed by a modification of the paper-disc plate method with a *Neurospora* sp. as the test organism (Westhead, 1961). The diameter of the zone of inhibition was plotted against the log concentration of the antifungal antibiotic over a range of 25 to 200 $\mu$g/ml. Plates with a single 6-ml seeded layer of modified Penassay agar (Difco), pH 7, were incubated 16 hr at 30 C. To make the antifungal antibiotic diffuse properly from the paper disc and produce a sharply defined zone of inhibition, a solvent mixture, containing 50% methanol and 50% polyethylene glycol 200, was used for preparing the dilutions of A272 for the standard curve, as well as for the dilutions of assay samples.

*Paper chromatography of A272.* A comparison of the chromatographic behavior of oligomycins A, B, and C with A272 is shown in Fig. 1. To obtain these bioautographs, known quantities of oligomycins A, B, C and A272 were chromatographed on dry Whatman #1 paper (19 cm × 46.5 cm). After evaporation of the solvent used in applying the samples to the respective lanes, the sheet was developed for 20 hr by descending chromatography in either solvent system 9 or 17. Solvent system 9 was composed of 75% water and 25% methyl acetone, which had been adjusted to pH 10.5 with $NH_4OH$ and then lowered to pH 7.5 with $H_3PO_4$. Solvent system 17 was 10% aqueous n-propanol. At the end of the development, the chromatograms were dried at room temperature. The position of the active components was located by bioautograph with *Fusarium moniliforme* M45-399, as the test organism.

*Isolation of A272.* Since paper chromatographic evidence had indicated a similarity of A272 to the oligomycins, a modification of the procedure described for the isolation of the oligomycins (Smith, Peterson, and McCoy, 1954) proved quite satisfactory for purifying A272. However, in the case of A272, the majority of the antifungal antibiotic was located in the mycelia. The following example will describe the procedure developed for isolating crystalline A272 from mycelia:

First, 50 liters of fermentation broth were mixed with Hyflo Super-Cel (5 g/100 ml) filter aid and were filtered on a Büchner funnel. The filtrate was discarded and the wet filter cake extracted three times by stirring 30 min with 12 liters 70% acetone. Each

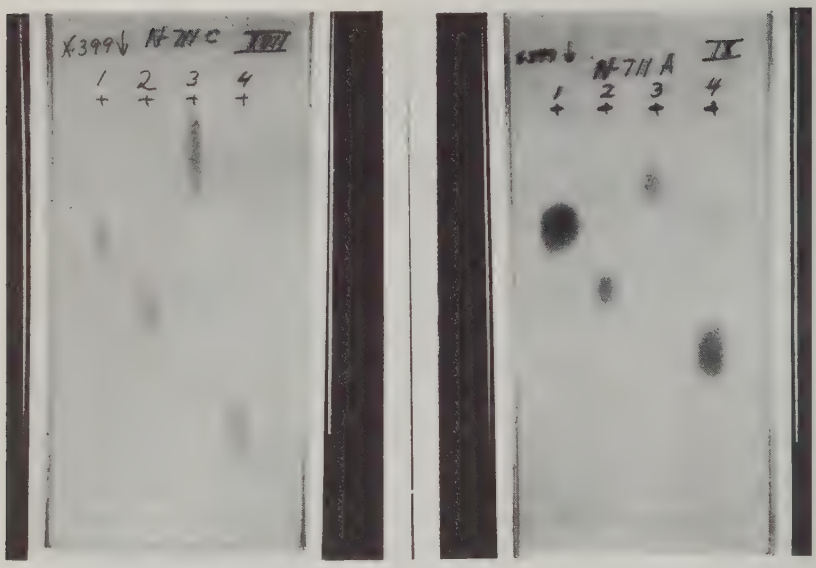


FIG. 1. *Bioautographs of oligomycins A, B, C, and A272. Run N-711A, solvent 9, 75% water-25% methylacetone, pH 7.5; lane 1, 0.5 μg oligomycin A; lane 2, 0.5 μg oligomycin B; lane 3, 0.25 μg oligomycin C; lane 4, 1 μg A272. Run N-711C, solvent 17, 10% aqueous n-propanol; lane 1, 0.3 μg oligomycin A; lane 2, 1.0 μg oligomycin B; lane 3, 0.5 μg oligomycin C; lane 4, 0.67 μg A272.*

extract was removed by filtration. After combining the extracts (32.5 liters), the acetone was removed under reduced pressure. The remaining aqueous concentrate (10.5 liters) was extracted three times with 3.5 liters of ethyl acetate per extraction. The spent aqueous phase was discarded. A viscous residue containing the A272 activity was obtained from the combined ethyl acetate extracts by removal of the solvent under reduced pressure. This residue was dissolved in 350 ml methanol, yielding a solution containing 137 mg solids/ml. The methanolic solution was stirred 30 min with 175 g (5%) Norite SG and was filtered. The filtrate containing the A272 activity was treated a second time with 5% Norite SG and filtered. The methanolic filtrate was concentrated to 150 ml and set in the refrigerator to crystallize. Several days later, 12.2 g of colorless crystalline A272, mp 124 to 126 C, were harvested. A second crop of slightly yellow crystals, 7.4 g, was subsequently harvested, mp 117 to 119 C. The over-all recovery from the initial acetone extracts to the crystalline A272 was 80%.

*Recrystallization of A272.* A272 may be recrystallized from either methanol-water or diethyl ether. For example, A272 (5 g, mp 126 C) was dissolved in a minimum of hot methanol, and water

was added slowly to incipient opalescence. After standing overnight in the refrigerator, 4.4 g of A272 were recovered, mp 127 to 128 C. The harvested crystals were usually washed lightly with a 1:1 mixture of diethyl ether and Skellysolve-B and dried under reduced pressure at room temperature. A similar product was obtained when diethyl ether was used as the solvent for recrystallization.

## Results

*Physical and chemical properties.* A272 is soluble in most of the common organic solvents, including lower alcohols, lower esters, ketones, chloroform, dimethyl formamide, and dimethylsulfoxide. It is only slightly soluble in diethyl ether and is relatively insoluble in water, Skellysolve-B, and Skellysolve-F.

A272 is stable in methanol-buffer solutions from pH 2 to 10 for several days and has shown little degradation in boiling organic solvents. It is stable for long periods of time when stored as dry crystalline material or when dissolved in suitable solvents.

Electrometric titration of A272 in 80% methanol or in 66% dimethylformamide indicated that there were no titratable groups in this compound. The ultraviolet-absorption spectrum in ethanol has a maximum at 225 mμ, $E^{1\%}_{1\ cm}$ = 445. The infrared absorption of A272 in chloroform solution is shown in Fig. 2. The marked similarity of this spectrum to the infrared spectra of oligomycins A, B, and C (Masamune et al., 1958) is to be noted. The optical rotation of A272 was $[\alpha]^{25}_{D}$ = -40.3° (c = 4.4, dioxane).

Single crystal X-ray diffraction measurements indicated that A272, crystallized from a methanol-water mixture, is monoclinic with lattice constants: a = 20.57 A, b = 11.10 A, and c = 11.30 A, $\beta$ = 71°, and a pycnometric density of 1.143 g/cc. The molecular weight calculated for four molecules per unit cell is 419.2 compared with a calculated molecular weight of 438.59.

Analysis calculated for $C_{25}H_{42}O_6$ was: C, 68.46%; H, 9.65%, O, 21.89%; 4C-$CH_3$, 13.68%; active hydrogen 6H, 1.38%. The following were found: C, 68.25%; H, 9.53%; O, 21.80%; C-$CH_3$, 13.66%, and active hydrogen 1.46%.

*Biological properties.* The in vitro antifungal spectrum of A272 was determined by plate (agar dilution) procedure. Penassay seed agar (Difco) was used in the agar dilution tests. Minimal concentrations of A272 for complete inhibition of fungi are shown in Table 1.

A272 did not inhibit, at 100 μg or less, gram-positive and gram-negative bacteria or species of *Candida, Hansenula, Saccharomyces, Schwanniomyces, Hanseniaspora,* and *Torulopis.* It is to be noted that A272 exhibited relatively high activity against several dermatophytic species in marked contrast to the lack of inhibition against dermatophytes (Marty and McCoy, 1959) reported for oligomycins A,

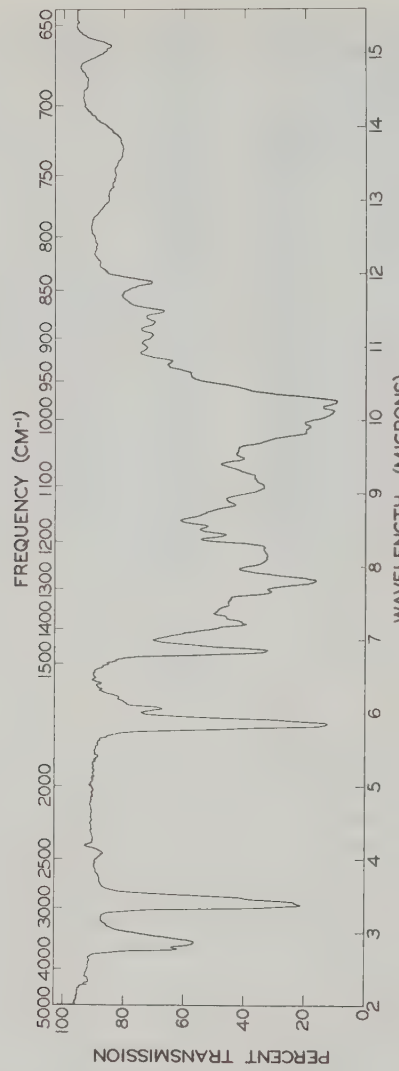


FIG. 2. *Infrared absorption spectrum of A272 in chloroform.*

B, and C. This difference in activity is not too surprising in view of the differences observed for oligomycins A, B, and C against several species of fungi (Marty and McCoy, 1959).

To determine if A272 is fungistatic or fungicidal, the organisms, *Ceratostomella ulmi*, *Glomerella cingulata*, and *Pullularia* sp. were exposed to two-fold serial dilutions of A272 from 0.2 to 100 $\mu$g/ml in Penassay broth (Difco). After these dilutions had incubated 24 hr,

plate counts were made of the surviving organisms. No significant changes were observed in total colony counts from controls, indicating that A272 is fungistatic.

TABLE 1. *Antifungal spectrum of A272 in vitro*

| Test organisms | Minimal inhibitory concentration at 72 hr $\mu g/ml$ |
|---|---|
| *Alternaria solani* G368 | 3.13 |
| *Aspergillus niger* M45-440 | 3.13 |
| *Botrytis cinerea* M45-298 | 1.56 |
| *Ceratostomella ulmi* M45-607 | 0.78 |
| *Colletotrichum lagenarium* G391 | 0.2 |
| *Colletotrichum pisi* G2 | 0.4 |
| *Collectotrichum phomoides* G277 | 0.4 |
| *Endoconidiophora fagacearum* G80 | 25.0 |
| *Epidermophyton floccosum* ATCC 10227 | 6.25 |
| *Fusarium moniliforme* M45-399 | 6.25 |
| *Fusarium oxysporium lycopersici* G24 | 6.25 |
| *Glomerella cingulata* G22 | 1.56 |
| *Helminthosporium sativum* G366 | 1.56 |
| *Microsporum canis* ATCC 10214 | 6.25 |
| *Microsporum audouini* ATCC 10216 | --- |
| *Pellicularia filamentosa* G422 | 25.0 |
| *Penicillium expansum* M45-883 | 6.25 |
| *Phomo pigmentovora* M45-467 | 3.13 |
| *Polyporus ostreatus* A6719 | 6.25 |
| *Pullularia* sp. M45-570 | 0.78 |
| *Pythium irregular* G389 | 0.4 |
| *Sclerotinia fructicola* G177 | 0.78 |
| *Trichophyton interdigitale* A19 | 3.13* |
| *Trichophyton rubrum* A20 | 1.56* |
| *Trichophyton mentagrophytes* ATCC 9129 | 25.0 |
| *Verticillium albo-atrum* G196 | 0.78 |

*Read at 48 hr.

The toxicity of A272 was tested in mice by both the oral and intraperitoneal routes. The $LD_{50}$ of A272 by oral administration was approximately 800 mg/kg; by intraperitoneal injection the $LD_{50}$ has varied from 18 to 74 mg/kg for crystalline preparations of similar purity. This variation in toxicity is being further investigated.

A272 applied topically as a 1% suspension or administered orally or parenterally was inactive against a cutaneous infection of *Microsporum canis* in guinea pigs.

A272 proved relatively ineffective when tested against the following plant diseases: early blight *(Alternaria solani)* of tomatoes, powdery mildew *(Erysiphe polygoni)* of beans, and *Fusarium* wilt *(Fusarium oxysporum f. tracheiphilum)* of cowpeas. The compound was completely ineffective against black stem rust *(Puccinia graminis tritici)* of wheat, the root knot nematode *(Meloidogyne incognita)* of

tomatoes, and as a general soil fungicide. Cotton seed infected with *Glomerella gossypii,* and beet, corn, and pea seeds infected with *Rhizoctonia solani* were not protected by application of A272.

## Discussion

Upon comparison of the data for A272 with the published data for oligomycins A, B, and C (Masamune et al., 1958; Marty and McCoy, 1959), many similarities may be noted. However, specific differences exist in paper chromatographic behavior, antifungal spectrum, toxicity, X-ray diffraction, optical activity, molecular formulae, and mp which indicate that A272 is a new antifungal antibiotic belonging to the oligomycin family.

The toxicity of A272 and the lack of in vivo activity suggest that this compound will not have useful application in human therapy. Similarly, the lack of activity against a variety of plant diseases eliminates this antifungal antibiotic as a useful agricultural chemical.

## Acknowledgments

The authors wish to express their appreciation to Sue Hughes and Constance Van Valer for technical assistance and to the many members of the Lilly Research Laboratories for their contributions to this work.

## Literature Cited

Marty, E. W., Jr., and E. McCoy. 1959. The chromatographic separation and biological properties of the oligomycins. Antibiotics & Chemotherapy 9:286-293.

Masamune, S., J. M. Sehgal, E. E. van Tamelen, F. M. Strong, and W. H. Peterson. 1958. Separation and preliminary characterization of oligomycins A, B, and C. J. Am. Chem. Soc. 80: 6092-6095.

Smith, R. M., W. H. Peterson, and E. McCoy. 1954. Oligomycin, a new antifungal antibiotic. Antibiotics & Chemotherapy 4:962-970.

Westhead, J. 1961. The assay of a highly water-insoluble antibiotic with *Neurospora.* M.S. Thesis, Butler University, Indianapolis, Ind.

# IN VITRO ANTIBACTERIAL POTENCY OF CHALCOMYCIN

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Chalcomycin, a fermentation product of an as yet unidentified *Streptomyces* species, was discovered in the laboratories of Parke, Davis and Company prior to 1959 (Kitto, *personal communication*). Isolated as a neutral compound which is not a salt, chalcomycin has a molecular weight of about 600. The chemical structure has not yet been fully worked out, but there are carbohydrate groupings in the molecule.

Early tests (Kitto, *personal communication)* disclosed no antibiotic effect on fungi, rickettsia, viruses, or protozoal parasites. Antibacterial antibiosis was most marked with various of the gram-positive cocci, including staphylococci, and was least apparent with gram-negative bacilli.

To define further the scope of antibacterial action of chalcomycin, the susceptibility of 391 bacterial isolates of human origin was determined in vitro by means of a tube dilution in broth method. Although the exact molecular weight of chalcomycin is not known, concentrations for susceptibility testing were prepared on the basis of 600 $\mu$g constituting 1 $\mu$mole. The isolates studied were representative of 12 bacterial groups, notable for frequent display of resistance to presently available antibacterial agents suitable for systemic usage (Table 1).

Also observed, but not directly concerned in assay of susceptibility, was the effect on chalcomycin of bacterial penicillinases and ethylene oxide gas. Staphylococci were, in addition, exposed to chalcomycin under conditions suitable for propagation of L forms.

## Materials and Methods

*Gram-negative bacilli and enterococci.* Arizona and Bethesda-Ballerup isolates were kindly supplied by Stanley Falkow (Newport Hospital, Newport, R.I.) and Philip R. Edwards (Communicable Disease Center, U.S. Public Health Service, Department of Health, Education and Welfare, Atlanta, Ga.). The remaining gram-negative

bacilli and the enterococci which were tested were isolated from patient specimens submitted to the Clinical Microbiology Laboratory of Salt Lake County General Hospital.

TABLE 1. *In vitro effect of chalcomycin on 12 groups of bacteria**

| Bacterial group | Number inhibited | Number tested | Chalcomycin |
|---|---|---|---|
| | | | *μM/ml* |
| *Escherichia coli* | 3 | 42 | 0.250 |
| *Klebsiella* sp. (*Aerobacter aerogenes*) | 0 | 23 | 0.250 |
| *Shigella* sp. | 0 | 5 | 0.250 |
| *Salmonella* sp. | 0 | 12 | 0.250 |
| Arizona | 0 | 5 | 0.250 |
| Bethesda-Ballerup | 0 | 5 | 0.250 |
| *Proteus mirabilis* | 0 | 18 | 0.250 |
| *Proteus morganii* | 0 | 15 | 0.250 |
| *Proteus rettgeri* | 0 | 5 | 0.250 |
| *Proteus vulgaris* | 0 | 10 | 0.250 |
| Providence bacilli | 0 | 15 | 0.250 |
| *Pseudomonas* sp. | 1 | 18 | 0.250 |
| *Enterococcus* | 0 | 13 | 0.250 |
| *Mycobacterium tuberculosis* (human) | 0 | 6 | 0.250 |
| *Mycobacterium* (unclassified) | 0 | 6 | 0.250 |
| *Staphylococcus aureus* | 192 | 193 | 0.100 |

*The chalcomycin concentration listed is the highest used for testing the susceptibility of the corresponding bacterial group.

For storage, each isolate was inoculated into 2 ml of semi-solid agar (commercial tryptic digest of casein-papaic digest of soybean broth base plus 0.4% agar) in a screw-capped tube (13 × 100 mm). After 24 hr of incubation at 37 C, the caps were sealed with Parafilm before the tubes were transferred to a freezer chest at -22 C.

In addition to testing for susceptibility to chalcomycin, each isolate was tested at the same time with benzylpenicillin, aminocarboxybutylpenicillin, dihydrostreptomycin, neomycin, kanamycin, polymyxin B, chloramphenicol, tetracycline, sulfadiazine, nitrofurantoin, and furaltadone. These agents were prepared in solutions of 0.0025 M, 0.001 M, 0.0001 M, and 0.00001 M concentrations in a synthetic broth medium modified from Toennies and Shockman (1953) by decrease in phosphate concentration to 0.06 M, omission of DL-alanine, and substitution of minerals and accessory growth substances described by Hoeprich (1955). Series of 13 × 100 mm screw-capped

tubes were prepared for testing by transfer of 0.1-ml portions of the stock solutions of the agents to be tested and were stored at -22 C until use.

Chalcomycin, Lot Rx X-7434, was supplied by William Kitto of Parke, Davis and Co., Ann Arbor, Mich. Potassium benzylpenicillin, dihydrostreptomycin sulfate, polymyxin B sulfate, chloramphenicol, and tetracycline were provided by Frederick C. Fink of Chas. Pfizer and Co., Brooklyn, N.Y. J. T. Sylvester of Abbott Laboratories, North Chicago, Ill., supplied disodium aminocarboxybutylpenicillin as Salmotin test powder, Lot 2085 CP. Neomycin sulfate and isonicotinic acid hydrazide were obtained from a commercial source. Bristol Laboratories, Inc., Syracuse, N.Y., supplied kanamycin sulfate. Charles H. Tracy of Eaton Laboratories, Norwich, N.Y., supplied sodium nitrofurantoin and furaltadone hydrochloride. Potassium phenoxymethylpenicillin, Lot 755838, was obtained from G. E. Maha of Eli Lilly and Co., Indianapolis, Ind.

Preparatory to testing, each isolate was grown out overnight in 10 ml of a synthetic broth medium inoculated from the frozen storage culture. Inoculations with approximately 10,000 bacteria in 0.9 ml of synthetic broth medium, incubation, interpretation, and testing to distinguish static from bactericidal effect were carried out as previously described (Hoeprich, 1961).

*Mycobacteria. Mycobacterium tuberculosis* H37rv and unclassified *Mycobacterium* species were obtained through the kindness of Ernest H. Runyon (Tuberculosis Research Laboratory, Veterans Administration Hospital, Salt Lake City, Utah). The patient (wild) strains of *M. tuberculosis* tested were isolated in the Clinical Microbiology Laboratory of the Salt Lake County General Hospital.

Each mycobacterial isolate was tested for susceptibility to chloramphenicol, dihydrostreptomycin, kanamycin, neomycin, and isonicotinic acid hydrazide, as well as chalcomycin. The following five concentrations of these agents were prepared in modified Proskauer-Beck broth medium (Youmans and Karlson, 1947); 0.0025 M, 0.001 M, 0.0005 M, 0.0001 M, and 0.00001 M. Samples (0.1 ml) were distributed to series of 13 × 100 mm screw-capped tubes and stored at -22 C until used.

For testing, each isolate was first grown out in modified Proskauer-Beck medium. The growth was homogenized and bacterial mass per unit volume of the resulting suspension was estimated by sedimenting a sample in a hematocrit tube (Wintrobe) and assigning 1 mg wet weight to each $mm^3$ of bacteria (Youmans and Karlson, 1947). The suspensions were then diluted in Proskauer-Beck medium so that the 0.9 ml of inoculum contained approximately 0.05 mg wet weight of mycobacteria.

Incubation at 37 C was continued until growth in a control tube, containing no chemotherapeutic agent, had proceeded to floccules layering the walls of the tube and surface of medium. Grossly

evident growth in test cultures was taken to indicate resistance. Tubes without evident growth were centrifuged at 1,850 × *g* for 30 min; the supernatant broth was removed and replaced with 1 ml of fresh Proskauer-Beck medium before incubation was resumed. Four weeks later, the presence or absence of growth was taken to distinguish static from bactericidal effect.

*Staphylococci.* Susceptibility testing of 193 tellurite-positive human-associated staphylococci (Hoeprich, Croft, and West, 1960) of community-wide (Salt Lake City) origin was carried out as previously described (Hoeprich, 1961*a*). Details regarding collection and storage of these isolates have been presented elsewhere (Hoeprich, 1961*b*). Some of these staphylococcal isolates were typed by determination of susceptibility to bacteriophages (Hoeprich, 1961*b*). Ability to produce penicillinase was assayed with each of these staphylococci (Hoeprich, 1961*a*).

At the same time susceptibility to chalcomycin was determined, the staphylococci were also tested with phenoxymethylpenicillin, benzylpenicillin, aminocarboxybutylpenicillin, dihydrostreptomycin, kanamycin, and neomycin.

*Exposure to penicillinase.* Parallel Gots plates (Gots, 1945) were prepared using $10^{-4}$ $\mu$M benzylpenicillin per ml in one set and $10^{-3}$ $\mu$M chalcomycin per ml in a second set to inhibit the *Sarcina lutea* indicator (*S. lutea* was kindly supplied by R. E. Rhodes, Microbiology Section, Smith, Kline and French Laboratories, Philadelphia, Pa.). A known penicillinase-producing staphylococcus was applied on one-third of the plate and 5,000 units crystalline commercial penicillinase was placed in a 5-mm (diameter) well cut into the agar of another third of the plate. Agar medium, without penicillin or chalcomycin but with *S. lutea*, was poured into the remaining third of the plate. Incubation in a moist air-candle jar was continued at 37 C for 5 days.

*Exposure to ethylene oxide gas.* Duplicate 0.05-g portions of chalcomycin were weighed and put into sterile 50-ml volumetric flasks. One flask was exposed to ethylene oxide gas (about 400 mg per liter) overnight, before solution in sterile distilled water. A total of 96 staphylococcal isolates were tested for susceptibility to both the ethylene oxide exposed and the nonexposed chalcomycin in the manner described.

*L form induction.* Wells, 5 mm in diameter, were cut in an agar medium suitable for growth of staphylococcal L forms (Marston, 1961). Two strains of surface-inoculated staphylococci were exposed to about 2.5 $\mu$M chalcomycin, 1.5 $\mu$M benzylpenicillin, and 25 $\mu$M cycloserine by addition of solutions of these antibiotics in 6% NaCl. The plates were inspected daily during 10 days of incubation at 37 C.

## Results

Both the gram-negative bacilli and the enterococci tested were so infrequently susceptible to chalcomycin that no further comment is indicated. None of the mycobacterial species exposed to chalcomycin was inhibited from growth. Chalcomycin did inhibit staphylococci at all concentrations employed (Table 1).

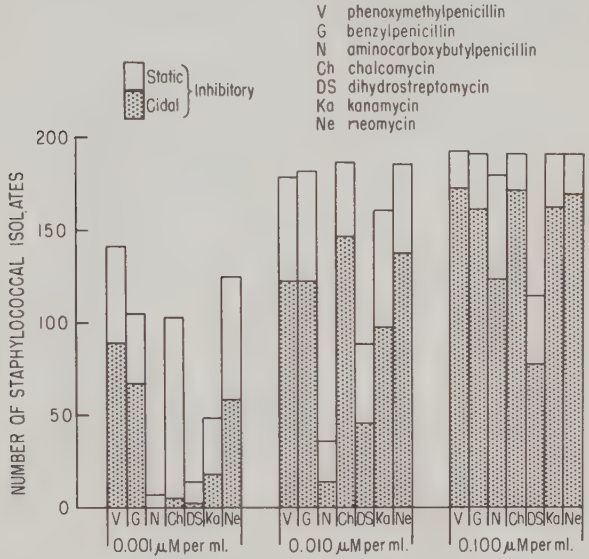


FIG. 1. *Total height of the columns corresponds to the numbers of staphylococcal isolates of the 193 tellurite-positive strains of clinical origin which were tested and were inhibited (bacteriostatic plus bactericidal effect) at each of the three test concentrations of the seven antibiotics. The stippled zones indicate the extent of bactericidal effect. At 0.001 μM per ml of each agent, chalcomycin was of about the same inhibitory potency as benzylpenicillin; phenoxymethylpenicillin and neomycin were significantly more often inhibitory. With the higher test concentrations, chalcomycin was not significantly more or less effective as an inhibitor of staphylococci than were phenoxymethylpenicillin, benzylpenicillin, and neomycin. Dihydrostreptomycin was uniformly less effective than chalcomycin. Although aminocarboxybutylpenicillin and kanamycin were less potent at lower concentrations, both were as inhibitory to staphylococci as chalcomycin at test concentrations of 0.1 μM per ml. Although chalcomycin was primarily bacteriostatic in action when present in a concentration of 0.001 μM per ml, a 10-fold increase in concentration was associated with a marked increase in bactericidal potency. Symbols: phenoxymethylpenicillin* (V); *benzylpenicillin* (G); *aminocarboxybutylpenicillin* (N); *chalcomycin* (Ch); *dihydrostreptomycin* (DS); *kanamycin* (Ka); *neomycin* (Ne).

Comparison of the antistaphylococcal potency of chalcomycin with the potency of the simultaneously tested phenoxymethylpenicillin,

benzylpenicillin, aminocarboxybutylpenicillin, dihydrostreptomycin, kanamycin, and neomycin is conveniently made in Fig. 1. Table 2 lists $\mu$M, $\mu$g, and bioassay unit equivalents of the test concentrations used. Over-all, chalcomycin was about as potent an inhibitor of staphylococci as benzylpenicillin. Although less potent than phenoxymethylpenicillin and neomycin, chalcomycin was more potent than aminocarboxybutylpenicillin, dihydrostreptomycin, and kanamycin. These differences in potency were most marked, to the point of statistical significance ($p \leq 0.050$), in the tests carried out using 0.001 $\mu$M of each agent per ml.

TABLE 2. *Concentrations of antibiotics used to determine the susceptibility of 200 clinical isolates of tellurite-positive staphylococci*

| Antibiotic | Molecular weight | $\mu$M, $\mu$g, and bioassay unit equivalent of test concentration per ml | | |
|---|---|---|---|---|
| | | 0.001 $\mu$M | 0.01 $\mu$M | 0.1 $\mu$M |
| Phenoxymethylpenicillin | 388.47 | 0.39 $\mu$g | 3.89 $\mu$g | 38.85 $\mu$g |
| Benzylpenicillin | 372.47 | 0.37 $\mu$g<br>0.59 units | 3.73 $\mu$g<br>5.88 units | 37.24 $\mu$g<br>58.82 units |
| Aminocarboxybutylpenicillin | 403.37 | 0.40 $\mu$g<br>0.27 units | 4.03 $\mu$g<br>2.66 units | 40.34 $\mu$g<br>26.62 units |
| Chalcomycin | 600* | 0.60 $\mu$g | 6.0 $\mu$g | 60.0 $\mu$g |
| Dihydrostreptomycin | 583.59 | 0.58 $\mu$g | 5.84 $\mu$g | 58.36 $\mu$g |
| Kanamycin | 484.50 | 0.48 $\mu$g | 4.85 $\mu$g | 48.45 $\mu$g |
| Neomycin | 590.63 | 0.59 $\mu$g | 5.93 $\mu$g | 59.26 $\mu$g |

*Approximate value.

At this concentration, chalcomycin was not often lethal. It was a significantly better bactericidal agent than aminocarboxybutylpenicillin, about equal to dihydrostreptomycin, but was significantly inferior to phenoxymethylpenicillin, benzylpenicillin, kanamycin, and neomycin as a killer of staphylococci.

When 10 times more of each agent was present (0.01 $\mu$M per ml), the over-all inhibitory relationships were not so much changed as was the bactericidal potency of chalcomycin. In terms of both bactericidal and inhibitory potency, chalcomycin was slightly, but not significantly, more potent than phenoxymethylpenicillin, benzylpenicillin, and neomycin; it was, however, still significantly more potent than aminocarboxybutylpenicillin, dihydrostreptomycin, and kanamycin.

A further 10-fold increase in concentration to 0.100 $\mu$M/ml left only dihydrostreptomycin behind as still significantly inferior to all other agents of the test group. Aminocarboxybutylpenicillin was significantly less often bactericidal, but was about as often inhibitory as chalcomycin and the remaining agents.

The possibility of coincidence of staphylococcal susceptibility or resistance of chalcomycin with either phenoxymethylpenicillin or neomycin was examined by isolate by isolate comparison of test results with the three agents at 0.001 μM/ml (Table 3). There was no evidence for coincidence of either susceptibility or resistance among chalcomycin, phenoxymethylpenicillin, and neomycin. With the small numbers of isolates concerned, no phage type predilection was discernible in relation to susceptibility or resistance with chalcomycin, phenoxymethylpenicillin, or neomycin. Penicillinase production was demonstrable with only 6 of the 17 isolates which were inhibited by 0.001 μM phenoxymethylpenicillin, but resistant to 0.001 μM/ml of either chalcomycin or neomycin. On the other hand, all of those eight isolates inhibited by chalcomycin alone were also penicillinase producers. In accord with these susceptibility data was the lack of reversal of chalcomycin inhibition of *S. lutea* in the setting of Gots plate exposure of chalcomycin to staphylococcal penicillinase and to commercial *Bacillus cereus* penicillinase (Fig. 2).

TABLE 3. *Phage typing and penicillinase test results with those staphylococci of 193 tellurite-positive, clinical isolates which were inhibited by or resistant to chalcomycin, phenoxymethylpenicillin, and neomycin at two test concentrations of 0.001 μM/ml*

| Staphylococcal isolates | | Number typable | Number studied | Lysed by phages 80 or 81 | Penicillinase-positive |
|---|---|---|---|---|---|
| Group* | Number | | | | |
| Entire test | 193 | 75 | 159 | 18 | 127 |
| Inhibited by chalcomycin | 8 | 2 | 6 | 1 | 8 |
| Inhibited by phenoxymethyl-penicillin | 17 | 6 | 9 | 0 | 6 |
| Inhibited by neomycin | 13 | 6 | 13 | 4 | 12 |
| Resistant to chalcomycin | 46 | 27 | 41 | 3 | 31 |
| Resistant to phenoxymethyl-penicillin | 14 | 6 | 12 | 3 | 12 |
| Resistant to neomycin | 20 | 5 | 15 | 1 | 12 |

*In each case of antibiotic inhibition, the group of isolates was resistant to the other two antibiotics. In each case of antibiotic resistance, the isolates were inhibited by the other two antibiotics.

With 96 isolates, parallel susceptibility testing was carried out using chalcomycin exposed to ethylene oxide gas and unexposed chalcomycin. Although there was no visible effect from ethylene oxide exposure, there was a statistically significant decrease ($0.02 > p > 0.01$) in the frequency of occurrence of a bactericidal effect from 0.01 μM/ml of chalcomycin treated with ethylene oxide gas (Table 4).

FIG. 2. *Agar medium containing Sarcina lutea (1:100 final dilution of a 24-hr-old broth culture) without added antibiotic agent was poured into the lower third sector of each plate. To the remaining two-third sectors, the same agar, seeded with S. lutea, was poured, after addition of $10^{-4}$ μM benzylpenicillin per ml for the left plate, $10^{-3}$ μM chalcomycin for the right plate. About 0.3 ml of a solution containing 15,000 units pencillinase derived from Bacillus cereus was added to each of the 5-mm diameter wells which had been cut after the agar had gelled. After a known penicillinase-producing strain of Staphylococcus aureus was swab inoculated (from a 24-hr-old broth culture) on the remaining third sector of each plate, both plates were incubated at 37 C in a moist air-candle jar for 5 days. Chalcomycin was not inactivated by either B. cereus or staphylococcal penicillinase, and benzylpenicillin was inactivated by both penicillinases.*

TABLE 4. *Comparison of antistaphylococcal potency of chalcomycin exposed and unexposed to 400 mg of ethylene oxide per liter, overnight at room temperature, using 92 clinical isolates of tellurite-positive staphylococci in parallel susceptibility tests*

| Chalcomycin | Ethylene oxide | Number inhibited | Number resistant | Number bactericidal | Number nonbacter-icidal |
|---|---|---|---|---|---|
| *μM/ml* | | | | | |
| 0.001 | Unexposed | 57 | 35 | 4 | 88 |
| 0.001 | Exposed | 48 | 44 | 3 | 89 |
| 0.01 | Unexposed | 89 | 3 | 68* | 24 |
| 0.01 | Exposed | 88 | 4 | 51* | 41 |
| 0.1 | Unexposed | 92 | 0 | 79 | 13 |
| 0.1 | Exposed | 92 | 0 | 76 | 16 |

*$0.01 > p > 0.005$.

Exposure of two staphylococcal isolates to chalcomycin, under culture conditions suitable for propagation of L forms (as attested by the development of L forms in the same test system under the influence of benxylpenicillin and cycloserine), did not result in the appearance of L forms of staphylococci.

## Discussion

Although not effective by in vitro test against a variety of gram-negative bacilli, enterococci, or *Mycobacterium* species, chalcomycin was a potent inhibitor of tellurite-positive staphylococci of human origin. In low concentrations, it was less often effective than either phenoxymethylpenicillin or neomycin and displayed little bactericidal capability. At higher concentrations, chalcomycin was far more effective, attaining statistical parity in potency with both phenoxymethylpenicillin and neomycin.

Several bits of evidence, in addition to microbial source and physical properties, lead to the conclusion that chalcomycin is a significantly different antibiotic from those concomitantly studied. There was no discernible pattern of cross susceptibility or resistance with other agents simultaneously tested against the same bacterial isolates. Exposure to two bacterial penicillinases did not render chalcomycin ineffective. Antistaphylococcal potency was apparently decreased on exposure to ethylene oxide gas, from which one may infer that groupings essential to antibiosis are susceptible to the alkylating attack of ethylene oxide (Phillips, 1957). In limited trial, chalcomycin failed to induce protoplast formation from staphylococci and it is not likely that interference with cell-wall synthesis is a mechanism of chalcomycin-mediated antibiosis.

## Acknowledgments

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It is a pleasure to acknowledge the able technical assistance of Elsa M. Vanderhooft and Gloria M. Bristow. Garth F. Croft and Ingeborg E. Nelson isolated many of the bacterial strains tested.

## Literature Cited

Gots, J. S. 1945. The detection of penicillinase-producing properties of microorganisms. Science 102:309.

Hoeprich, P. D. 1955. $C^{14}$ labelling of *Diplococcus pneumoniae*. J. Bacteriol. 69:682-689.

Hoeprich, P. D., G. F. Croft, and L. M. West, II. 1960. Tellurite reduction as an indicator of potentially pathogenic staphylococci. J. Lab. Clin. Med. 55:120-128.

Hoeprich, P. D. 1961*a*. Side chain stereoisomerism and anti-staphylococcal potency of penicillins. J. Clin. Invest. 40:783-793.

Hoeprich, P. D. 1961*b*. Intracommunity admixture of human associated staphylococci. J. Lab. Clin. Med. 57:784-806.

Marston, J. 1961. Observations on L forms of staphylococci. J. Infectious Diseases **108**:75-84.

Phillips, C. R. 1957. Gaseous sterilization, p. 754-761. *In* G. F. Reddish, [ed.], Antiseptics, disinfectants, fungicides and sterilization, 2nd ed. Lea and Febiger, Philadelphia.

Toennies, G., and G. D. Shockman. 1953. Quantitative amino acid assimilation in homofermentative metabolism. Arch. Biochem. Biophys. **45**:447-458.

Youmans, G. P., and A. G. Karlson. 1947. Streptomycin sensitivity of tubercle bacilli. Am. Rev. Tuberc. **55**:529-535.

# BA-6903, A NEW ANTIBIOTIC

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In the course of our antibiotic screening program, an unidentified species of *Streptomyces* was isolated. The culture filtrates of this species exhibited activity against gram-positive bacteria and some protozoa. The active principle responsible for this was isolated as a crystalline compound, and its isolation and characterization are the subject of this paper.

## Materials and Methods

*Fermentation.* The organism grows well in submerged culture in the usual complex natural organic media. One of these employed for the fermentation consisted of the following components (in grams per liter): glucose, 10; distiller's solubles, 15; curbay BG, 5; glycerol, 5; and sodium chloride, 2. The medium was adjusted to a pH of 6.8 to 7.2, autoclaved for 45 min, and inoculated with a 48-hr-old vegetative culture of the organism grown in the same medium. The progress of the fermentation was followed by *Bacillus subtilis* plate assay and the beer was harvested at about 60 to 80 hr.

*Isolation.* It was found that the antibiotic activity was stable in the pH range of 2 to 10 for several hours, and filtration at the pH range of the broth gave only a partial recovery in the filtrate. The beer was, therefore, first adjusted to pH 9.0, stirred for 30 min, and filtered. Extraction of the acidified broth with solvents such as *n*-butanol, ethyl acetate, or methyl isobutyl ketone gave activity in the solvent layer in decreasing order. The broth was extracted with *n*-butanol; the extract was concentrated and shaken with ethyl acetate. The solvent layer, after concentration, was then passed through a column of acid-washed alumina. The antibiotic was adsorbed on the column and could be eluted with 5 to 10% methanol in ethyl acetate. The material was recovered and subjected to countercurrent distribution in the system chloroform-methanol-water-ligroin (3:3:1:1) in a 100-tube automatic apparatus. Based on optical density at 275 m$\mu$ and activity against *B. subtilis*, the main band was in tubes 20 to 40. The compound was recovered from the appropriate fractions and crystallized twice from acetone-ethyl acetate.

## Results

BA-6903 is a bright yellow crystalline solid which melts at 200 to 202 C. Analysis: $C_{23}H_{30}O_{10}$; calculated: C, 59.22; H, 6.48. Found: C, 59.74; H, 6.18.

In the system already referred to, the pure material behaves as a homogeneous substance in countercurrent distribution (Fig. 1).

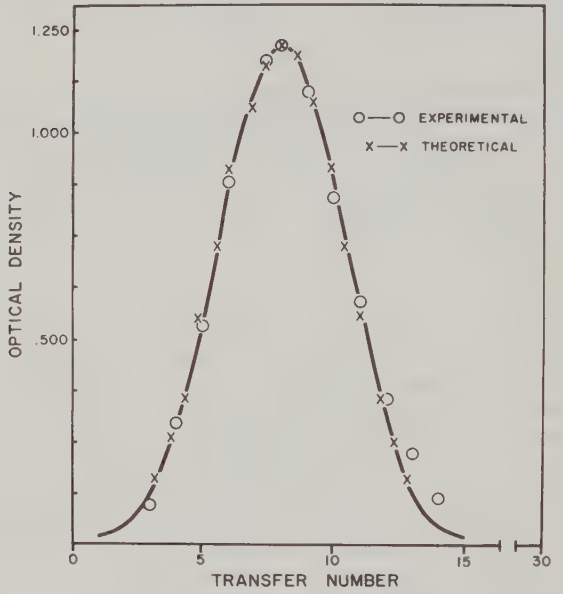


FIG. 1. *Countercurrent distribution of BA-6903.*

The ultraviolet spectrum of BA-6903 in methanol is shown in Fig. 2. It shows maxima at 232, 275, and 363 mμ with specific extinction values ($E^{1\%}_{1\,cm}$) of 308, 336, and 168, respectively. The infrared spectrum, run as a potassium bromide pellet (Fig. 3), shows strong absorption in the hydroxyl, carbonyl, and aromatic regions.

BA-6903 is moderately soluble in lower alcohols, acetone, ethyl acetate, and chloroform. It is only slightly soluble in water but dissolves more readily in dilute aqueous sodium hydroxide. It appears to be stable for 24 hr in 1 N sodium hydroxide at room temperature. The compound gives a deep greenish-brown color with ferric chloride and behaves as a weak acid.

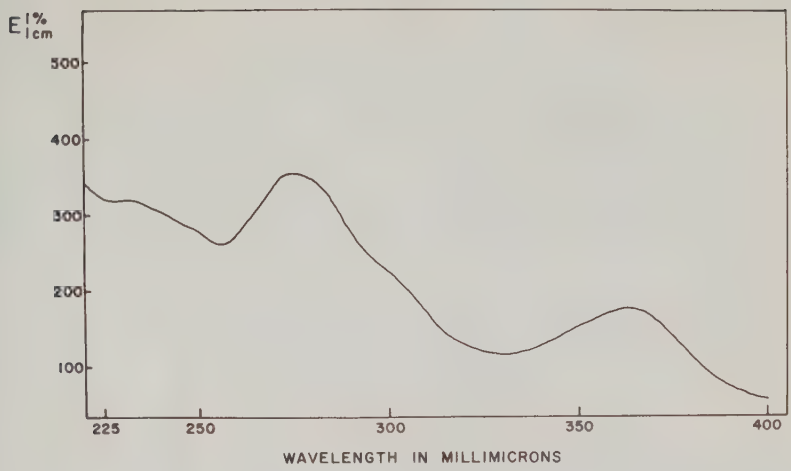


FIG. 2. *Ultraviolet spectrum of BA-6903.*

TABLE 1. *Biological activity of BA-6903*

| Organism | Minimal inhibitory concentration |
|---|---|
| | *μg/ml* |
| *Staphylococcus aureus* | 1.56 |
| *Streptococcus pyogenes* | 1.56 |
| *Diplococcus pneumoniae* | 6.25 |
| *Aerobacter aerogenes* | 100 |
| *Escherichia coli* | 100 |
| *Salmonella typhosa* | 100 |
| *Trichomonas vaginalis* | 0.5 |

The biological activity of BA-6903 is shown in Table 1. Its antibacterial activity is chiefly confined to gram-positive bacteria.

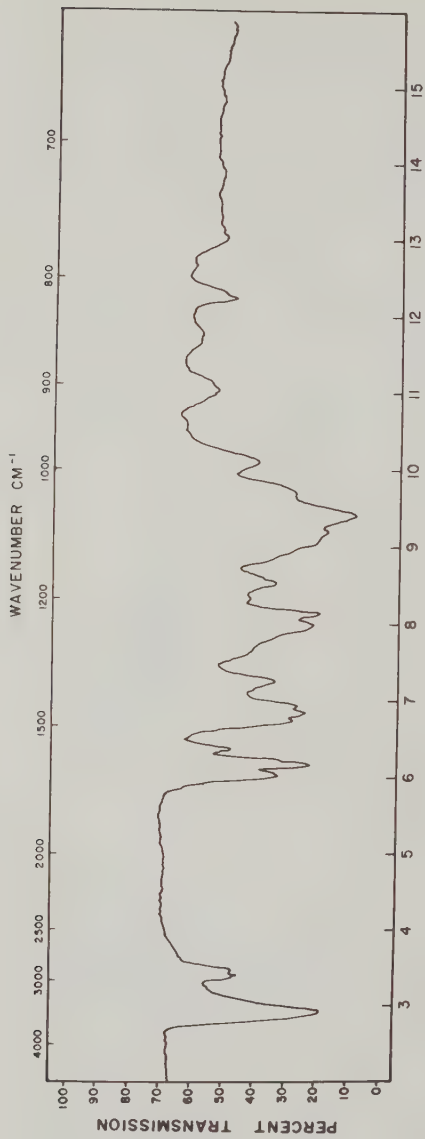


FIG. 3. *Infrared spectrum of BA-6903.*

# M-141, A NEW ANTIBIOTIC

## I. Antimicrobial Properties, Identity with Actinospectacin, and Production by *Streptomyces flavopersicus*, Sp. n.

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A new antibiotic, M-141, was isolated in our laboratories from a streptomycete obtained from a soil sample collected near Highland, Ind. The producing organism, *Streptomyces flavopersicus*, sp. n., is on deposit with the Northern Utilization Research and Development Division, U.S. Department of Agriculture, as NRRL B-2820.

Similarities noted between the biological and paper chromatographic characteristics of M-141 and those published for actinospectacin by Mason, Deitz, and Smith (1961) were confirmed by comparison of their sulfate salts. Physical chemical data provided further confirmation of their identity.

This paper describes the taxonomic characteristics of *S. flavopersicus*, production of antibiotic M-141, and its biological properties including comparisons with actinospectacin. Isolation and physical chemical data by Sinclair and Winfield (1962) and pharmacological properties by Hwang et al. (1962) are given in the following papers of this series.

### Materials and Methods

*Taxonomy.* Standard media used in this study correspond to those found in many taxonomic studies reported in the literature on *Streptomyces*. Morphological and color observations as well as carbohydrate assimilation studies were done from growth at 28 C on 20.0 ml of agar medium per 100 mm pressed-bottom petri plate. These media were atuoclaved 20 min at 121 C in 200-ml quantities in 500-ml Erlenmeyer flasks without premelting the agar. Tests for nitrite were done on agar slants. Peeled potato wedges, washed overnight in running water, were autoclaved in standard 18-mm test tubes. Plain gelatin solution, incubated at 24 C, and litmus milk

were in 7-ml amounts in 18-mm test tubes. With the exception of soluble starch, carbohydrate solutions were added aseptically to the basal medium of Pridham and Gottlieb (1948) after autoclaving. Preserved human bank blood was used to observe hemolysis.

Determinations of color were made with the aid of removable plastic color chips of the Color Harmony Manual (Jacobson, Granville, and Foss, 1948). Color names corresponding to the coded color chips are those of the Inter-Society Color Council, National Bureau of Standards Method of Designating Colors (1955). Use of the color manual and the ISCC-NBS method of naming colors follows the recommendation of the Committee on Color Standards of the Subcommittee on Taxonomy of Actinomyces of the American Society for Microbiology (*unpublished data*).

## Results and Discussion

Observations of growth habit, gross morphology, color, and physiological reactions are given in Table 1. Spore color was uniform

TABLE 1. *Gross taxonomic characteristics of Streptomyces flavopersicus at 15 days, 28 C, unless otherwise specified**

| Medium | Growth | Aerial mycelium | Sporulation | Substratal mycelium | Diffusible pigment, notes |
|---|---|---|---|---|---|
| Waksman's nutrient agar | Abundant. Isolated colonies with occasional diametric, rare radial wrinkling in convex centers, spreading over agar to 5 to 7 mm | Full. White (b) to Light Gray (c) | Virtually absent to 21 days | Dark Yellow (2 ne) | None |
| Carvajal oatmeal agar | Abundant. Isolated colonies extend flatly over agar to 7 mm from 3 mm convex center | Heavy, fluffy, Pale Orange Yellow (3 ca, 3 ea) with raised tufts of Yellowish Pink (4 ca) | Heavy | Grayish Yellow (2 gc) | None. Note: partial clearing, 8.0 mm zone around 3 mm colony 7 days |
| Czapek's dextrose agar | Moderate to good, unwrinkled growth. Isolated colonies convex 3–4 mm | Full. White (a) to slight pinkish tint | Poor through 21 days | Pale Yellow (2 fb) | None |
| Dextrose asparagine agar | Good to abundant. Deeply convex 3 mm centers of isolated colonies spreading to 7 mm arborescent to feathery edge | Full, fluffy, Pale Orange Yellow (3 ca) | Good | Pale to Moderate Yellow (2 fb, 2 kb) | None |
| Calcium malate agar | Moderate | Abundant White (a) | None through 21 days | – | None. Note: no visible digestion of malate 3, 7, 15, 21 days |
| Yeast extract malt extract agar | Abundant. 7 mm isolated colonies with slight 2 mm central depression | Full, fluffy. Pale Orange Yellow (3 ca) | Good | Moderate Yellow (2 ic) | None |
| Tomato paste oatmeal agar | Abundant growth with grossly tufted aerial by 3 days. Mycelium extends deeply into agar beyond surface limit of 6 mm isolated convex colonies | Abundant, fluffy. Pale Orange Yellow (3 ca) | Heavy | Dark Orange Yellow (3 pe) | None |
| Potato | Abundant. Pale Yellow Green (1ca) nonaerial growth at bottom, full aerial at top | Pale Orange Yellow (3 ca) 21 days | Good | – | Dark Grayish Yellow (2 ic) discoloration |
| Synthetic nitrate agar | Good | Full, White (a) | None to 21 days | Yellow | None. Nitrite positive |

*Additional observations: milk is completely peptonized without coagulation, pH 8.0; plain gelatin is rapidly liquefied, growth settles, reddish brown pigment in aerated portion above flocculent mycelium; blood is hemolized by nonaerial growth; starch is rapidly hydrolized by abundantly sporulating growth, no soluble pigment.

on all media producing good sporulation. This uniformity of color carried over to growth on agar plates in the carbon-utilization studies. Spore colors noted in the latter were pale orange yellow (3 ca) and pale to light yellowish pink (4 ca, 4 ea, 5 cb); these are pastel shades. Reverse mycelium on the carbon plates were light yellow (2 ea) to grayish yellow (2 ec, 2 gc).

Table 2 shows the carbon-utilization pattern of *S. flavopersicus*. Most of the carbon sources which were utilized also fostered sporulation in this basal medium.

TABLE 2. *Assimilation of carbon sources by Streptomyces flavopersicus**

| Substrate | Assimilation | Substrate | Assimilation |
|---|---|---|---|
| Xylose | + | Soluble starch | + |
| Arabinose | – | Cellulose† | – |
| Rhamnose | – | Salicin | + |
| Glucose | + | Glycerol | + |
| Galactose | + | Mannitol | – |
| Mannose | + | Dulcitol | + |
| Fructose | + | Inositol | + |
| Sorbose | – | Sorbitol | – |
| Sucrose | – | Sodium citrate | + |
| Lactose | + | Sodium lactate | + |
| Maltose | + | Sodium succinate | + |
| Cellobiose | + | Sodium acetate | + |
| Raffinose | – | Sodium-potassium tartrate | – |

*On the basal medium of Pridham and Gottlieb (1948).

†Filter paper strips applied to agar surface after inoculation.

Microscope observations were made on all media producing sporulation. The micromorphology was consistent with the following characteristics observed on the basal medium of Pridham and Gottlieb (1948) with ducitol as the carbon substrate. The mycelium is nonseptate. The fruiting structure is biverticillate, spore chains being produced in whorls at the ends of short branches arising from a common locus around the principal aerial mycelial filament. These branches of the primary verticil usually number from two to four. Single branches bearing spore chains singly or in tufts are not observed, and monoverticillate structure of the fruiting mycelium is not found. The individual spore chains of medium length are found in hooks or canes, open loops, and loose coils. Tight coils or fists and true corkscrew spirals are not observed. Spores are bacillary with squarely blunt ends, rarely ovoid, never globose. Typically they appear to be two to three times as long as thick with straight sides. Morphological characteristics at 100 diameters are shown in Fig. 1.

Electron microscopy confirmed the features noted above. Figure 2, at 14,000 diameters after osmic fixation, shows details of branching of primary verticils as well as the bacillary to cylindrical and occasional ovoid morphology of the spores. These spores are of the

FIG. 1. *Streptomyces flavopersicus, gross micromorphology of sporulating mycelium, 100 ×.*

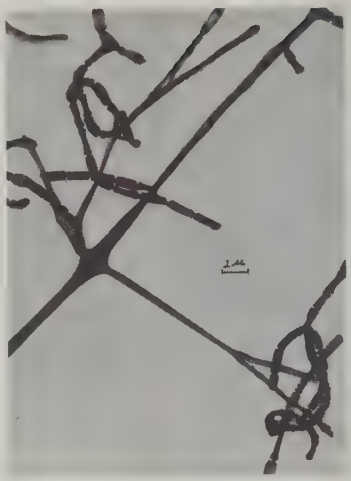


FIG. 2. *Streptomyces flavopersicus, detail of sporulating mycelium. Electron micrograph, 14,000 ×.*

smooth type free of spines, ridges, or convolutions although minute striations show in shadowed casts at 28,000 diameters in Fig. 3. The main filaments of aerial mycelium are 0.3 to 0.4 μ in diameter thickening to 0.5 μ adjacent to the loci of primary verticils. Spore size from electron micrographs average 0.4 × 1.1 μ with a range of 0.3 to 0.5 μ in diameter and 0.9 to 1.3 μ in length.

FIG. 3. *Streptomyces flavopersicus, spores. Electron micrograph, 28,000 ×.*

*S. flavopersicus* appears to be most closely related to *S. netropsis* by spore color and morphology of fruiting bodies. It differs from this species in lack of brown diffusible pigment produced by *S. netropsis* on a number of media, color of reverse mycelium, liquefaction of plain gelatin, action on milk, positive nitrite test from nitrate, and carbon-utilization pattern. Reference on *S. netropsis* is made to Finlay and Sobin (1952) and a cooperative study of streptomyces by the Subcommittee on Taxonomy of Actinomyces

of the American Society for Microbiology, distributed in mimeographed form at the annual meeting of the Society in 1959.

*S. flavopersicus* also differs from *S. cinnamomeus* which it resembles somewhat in spore color and lack of diffusible pigment. The principal differences are in micromorphology of spore chains and spores, action on milk, nitrite test, and carbon-utilization pattern. Reference to *S. cinnamomeus* f. *cinnamomeus*, NRRL B-1285, was made by Dvonch et al. (1954) and to *S. cinnamomeus* f. *azacoluta*, NRRL B-1699, in the cooperative study on streptomyces mentioned above.

Using the guide of Pridham, Hesseltine, and Benedict (1958), *S. flavopersicus* is placed in the Red series, but in section or subsection Biverticillus-Retinaculum Apertum which we propose as an amendment to the Pridham grouping. Section Biverticillus-Rectus Flexibilis is proposed for those species possessing tufts of short stiff to flexuous spore chains.

The specific name of *S. flavopersicus* is derived from the latin *flavus* "of a bright yellow-like gold" to describe the reverse mycelium color on clear agar media and the latin *color persicus* "of a peach color." The combined word describes the color of the mature spore mass.

*Production of antibiotic M-141.* Satisfactory yields of antibiotic M-141 are obtained in a variety of media utilizing soybean meal as the principal nitrogen source. Fermentation broths also contain a minor amount of another as yet unidentified antibiotic. In 200-liter fermentors at an operating volume of 120 liters, a medium composed of soybean meal, 20.0 g; cerelose, 25.0 g; NaCl, 5.0 g; $CaCo_3$, 1.0 g; soybean oil, 5.0 g; and a silicone antifoam, 1.0 g per liter, with pH unadjusted, is satisfactory. Additional antifoam during fermentation is unnecessary.

*Biological activity in vitro.* Antibiotic M-141 is active against a variety of gram-positive and gram-negative bacteria and *Actinomyces bovis*. Table 3 shows the antimicrobial spectrum of the crystalline dihydrochloride pentahydrate. Except where indicated, the medium is tryptose phosphate broth (pH 7.3) with readings at 48 hr. These data indicate that the antibiotic is somewhat weak in vitro and variable between strains of the same species.

Resistance to antibiotic M-141 develops rapidly with *Salmonella enteritidis*, *S. typhimurium*, *Pseudomonas aeruginosa* BMH #10, *Staphylococcus aureus*, and *S. epidermidis* ATCC 3519. The tube dilution transfer method in tryptose phosphate broth (pH 7.3) was used for these determinations.

Antibiotic M-141 is primarily bacteriostatic. The ratios between the minimal inhibitory concentration and minimal bactericidal concentration by the method of Grundy, Alford, and Sylvester (1959) are 16:1 and $>$ 32:1 with *S. epidermidis*, ATCC 3519, and *Escherichia coli*, ATCC 6880, respectively.

TABLE 3. *Antimicrobial spectrum of antibiotic M-141**

| Organism | MIC | Organism | MIC |
|---|---|---|---|
| | μg | | μg |
| *Actinomyces bovis* | 7.5 | *Proteus mirabilis*, BMH #9 | 60 |
| *Aerobacter aerogenes* | > 60 | *Proteus vulgaris*, JJ | 240 |
| *Aspergillus niger* | > 60 | *Pseudomonas aeruginosa*, ATCC 10145 | > 60 |
| *Bacillus subtilis* | 30 | *Pseudomonas aeruginosa*, BMH #10 | 18 |
| *Candida albicans* | > 60 | *Saccharomyces cerevisiae* | > 60 |
| *Chaetomium globosum* | > 60 | *Salmonella enteritidis* | 30 |
| *Clostridium sporogenes* | > 240 | *Salmonella typhimurium* | 60 |
| *Corynebacterium* sp. | 30 | *Sarcina lutea* | 30 |
| *Diplococcus pneumoniae* | 30 | *Shigella sonnei* | 45 |
| *Escherichia coli*, ATCC 6880 | 15 | *Staphylococcus aureus*, 209 P | 60 |
| *Escherichia coli*, Juhl | 30 | *Staphylococcus aureus*, Smith | 120 |
| *Klebsiella pneumoniae* | 15 | *Staphylococcus epidermidis*, ATCC 3519 | 60 |
| *Lactobacillus casei* | 15 | *Streptococcus faecalis* | > 60 |
| *Neisseria catarrhalis* | 7.5 | *Streptococcus pyogenes*, C 203 | 30 |
| *Pasteurella multocida* | 15 | | |

*In μg of base at 48 hr. Media: BHI broth - *S. faecalis, S. pyogenes;* BHI and 20% bovine serum - *Corynebacterium, D. pneumoniae;* Sabouraud broth - yeasts and molds; thioglycolate broth - *A. bovis, C. sporogenes;* tomato juice broth - *L. casei;* tryptone, glucose, yeast extract broth - *N. catarrhalis;* tryptose phosphate broth - all others. Sabouraud broth, pH 5.6; other media, pH 7.2 to 7.4.

*In vitro comparison with actinospectacin.* Bacterial resistance to actinospectacin develops to the same degree and in the same manner as to M-141. Complete cross resistance of the respective sulfate salts is shown in Table 4.

TABLE 4. *Cross resistance of M-141 sulfate with actinospectacin sulfate in brain heart infusion broth, pH 7.4, 48 hr*

| Organism | Minimal inhibitory level | |
|---|---|---|
| | M-141 | Actinospectacin |
| | μg/ml | μg/ml |
| *Staphylococcus aureus*, parent | 150 | 150 |
| *S. aureus*, M-141 R* | 1600 | 1600 |
| *S. aureus*, actinospectacin R | 1600 | 1600 |
| *Escherichia coli*, parent | 50 | 75 |
| *E. coli*, M-141 R | 1600 | 1600 |
| *E. coli*, actinospectacin R | 1600 | 1600 |

*R = Resistance induced in tryptose phosphate broth (pH 7.3).

A marked effect of pH on the in vitro activity of antibiotic M-141 has been observed. Table 5 shows this effect in a comparison of the sulfate salts of M-141 and actinospectacin. Both show the same increase in activity with increasing alkalinity.

*Assay method.* Antibiotic M-141 is conveniently assayed by the cylinder plate method using *E. coli*, ATCC 26, as the test organism. Antibiotic assay agar no. 5 (Difco) is used with the addition of 0.2%

TABLE 5. *Effect of pH on activity of M-141 sulfate and actinospectacin sulfate in brain heart infusion broth*

| Organism | MIC in μg/ml at 48 hr | | | | | |
|---|---|---|---|---|---|---|
| | M-141 | | | Actinospectacin | | |
| | pH 6.3 | pH 7.6 | pH 8.4 | pH 6.3 | pH 7.6 | pH 8.4 |
| *E. coli* | >800 | 50 | 12.5 | 400 | 50 | 12.5 |
| *S. typhimurium* | >800 | 200 | 25.0 | >800 | 100 | 25.0 |
| *P. aeruginosa* BMH #10 | >800 | 200 | 50.0 | >800 | 200 | 50.0 |
| *S. aureus* | >800 | 100 | 12.5 | 800 | 50 | 12.5 |
| *S. faecalis* | >800 | 400 | 50.0 | 800 | 400 | 50.0 |

glucose which serves to sharpen the edges of inhibition zones. A single 7.0-ml layer of seeded agar is used in standard 100-mm pressed-bottom petri plates. The diluent for the assay is 1.0% phosphate buffer (pH 8.0), and plates are incubated at 24 C for 16 to 17 hr after preliminary refrigeration for 2 hr. The sensitivity of the assay is approximately 7 μg of base.

*Antibacterial in vivo studies.* The cyrstalline dihydrochloride of antibiotic M-141 is active in CF-1 mice against a variety of infections (Table 6). The antibiotic was administered at 1, 3, 5, and 7 hr postinfection. The $CD_{50}$ values (Litchfield and Wilcoxon, 1949) are in terms of weight of free base and are a combination of several experiments. In vitro activities against the infecting organisms are included for comparison. The antibiotic is considerably more active intramuscularly than would be expected from its in vitro activity. The large doses required when the antibiotic is given orally indicate poor absorption.

TABLE 6. *In vivo protection of antibiotic M-141 in CF-1 mice in terms of free base*

| Infection | Dose × $LD_{50}$ | Route* | $CD_{50}$ | MIC in vitro |
|---|---|---|---|---|
| | | | *mg/kg* | *μg/ml* |
| *E. coli*, Juhl | 100 | IM | 6.3 (4.8-8.2)† | 30 |
| | 100 | Oral | 324 (287-366) | |
| *P. vulgaris*, JJ | 10-100 | IM | 31 (26-37) | 240 |
| | 10-100 | Oral | 450 (395-513) | |
| *P. mirabilis*, BMH #9 | 10-100 | IM | 6.0 (4.7-7.7) | 60 |
| | 10-100 | Oral | 288 | |
| *S. aureus*, Smith | 10-100 | IM | 9.0 | 120 |
| | 10-100 | Oral | 492 (447-541) | |
| *S. pyogenes*, C 203 | 10-100 | IM | 9.0 | 30 |
| | 10-100 | Oral | 576 | |
| *D. pneumoniae* | 100-1000 | IM | 34 (30-38) | 30 |
| | 100-1000 | Oral | >576 | |

*Medication: 1, 3, 5, 7 hr postinfection.
†Numbers in parentheses indicate 95% confidence limits.

*Antiparasitic in vivo studies.* At 120 mg/kg of feed, dihydrochloride M-141 is very active against coccidiosis (*Eimeria tenella*) in chicks. Mouse pinworm (*Syphacia obvelata*) is controlled by four daily doses of 300 mg/kg but not by 100 mg/kg doses. It is inactive against experimental schistosomiasis (*Schistosoma mansoni*) in mice at five daily intraperitoneal doses of 200 mg/kg.

## Literature Cited

Dvonch, W., O. L. Shotwell, R. G. Benedict, T. G. Pridham, and L. A. Lindenfelser. 1954. Further studies on cinnamycin, a polypeptide antibiotic. Antibiotics & Chemotherapy 4:1135-1142.

Finlay, A. C., and B. A. Sobin. 1952. Netropsin and process for its production. U. S. Patent 2,586,762.

Grundy, W. E., E. F. Alford, and J. C. Sylvester. 1959. Antibiotic bactericidal studies. I. Effect of methods on apparent bactericidal concentrations. Antibiotics Ann. 1958-1959, p. 847-854.

Hwang, K., L. Goen, H. E. Johnson, W. W. Hunter, and P. Cugier. 1962. M-141, a new antibiotic. III. Pharmacology. Antimicrobial Agents and Chemotherapy - 1961. p. 507-515.

Jacobsen, R., W. C. Granville, and C. E. Foss. 1948. Color harmony manual. 3rd ed. Container Corporation of America, Chicago.

Litchfield, J. T., Jr., and F. Wilcoxon. 1949. A simplified method of evaluating dose-effect experiments. J. Pharmacol. Exptl. Therap. 96:99-113.

Mason, D. J., A. Dietz, and A. R. Smith. 1961. Actinospectacin, a new antibiotic. I. Discovery and biological properties. Antibiotics & Chemotherapy 2:118-122.

National Bureau of Standards. 1955. The ISCC-NBS method of designating colors and a dictionary of color names. Circular no. 553. U. S. Govt. Print. Off., Washington, D. C.

Pridham, T. G., and D. Gottlieb. 1948. The utilization of carbon compounds by some Actinomycetales as an aid for species determination. J. Bacteriol. 56:107-114.

Pridham, T. G., C. W. Hesseltine, and R. G. Benedict. 1958. A guide for the classification of streptomycetes according to selected groups. Appl. Microbiol. 6:52-79.

Sinclair, A. C., and A. F. Winfield. 1962. M-141, a new antibiotic. II. Isolation and characterization and identity with actinospectacin. Antimicrobial Agents and Chemotherapy - 1961, p. 503-506.

# M-141, A NEW ANTIBIOTIC
## II. Isolation and Characterization and Identity with Actinospectacin

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The cultivation of *Streptomyces flavopersicus* to produce the new antibiotic M-141 has been described by Oliver et al. (1962). Several antibiotics can be demonstrated in the culture filtrate by the techniques of paper chromatography but the one of current interest is a fairly strong base which is removed from the beer by cation-exchange resins and eluted with any of a variety of acids to yield the desired salt. We have succeeded in crystallizing only the hydrochloride and sulfate salts.

A typical recovery procedure is as follows. The whole beer is treated with enough oxalic acid to precipitate the calcium in the medium, adjusting the pH to 6 with ammonium hydroxide. After filtering with the help of a filter aid, the clarified beer is passed over a column of carboxylic acid type ion-exchange resin in the sodium form. One liter of resin (Rohm and Haas IRC 50) in the sodium form holds about 18 g of M-141 base. After washing the column with water, the antibiotic is eluted with 0.5 N hydrochloric acid, collecting fractions and adjusting to pH 6. The fractions with high biological activity are combined and evaporated to a small volume. The antibiotic precipitates along with sodium chloride. The precipitate is extracted with methanol, which dissolves the antibiotic and leaves most of the sodium chloride behind. The methanol is evaporated with addition of water toward the end, and the antibiotic crystallizes from the cooled concentrated solution. The product thus obtained is at least 85% pure, but it is usually necessary to redissolve it in methanol and decolorize with carbon before recrystallizing. Instead of crystallizing the antibiotic from a concentrated water solution, one can add acetone to a dilute water solution.

The antibiotic base and its salts are very soluble in water and pyridine, moderately soluble in methanol, and almost insoluble in other common organic solvents.

The recrystallized hydrochloride salt is obtained in the form of long, narrow, sharp-pointed crystals. In crossed polarized light the crystals show many interference colors, high birefringence, and sharp parallel extinction.

Elemental analysis, titration data, and loss on drying fit the formula $C_{14}H_{26\text{-}28}O_7N_2 \cdot 2HCl \cdot 5H_2O$. On heating, the crystals lose water beginning at about 80 C and melt at about 210 C with decomposition. In water solution, the hydrochloride is dextrorotatory at the D line: $[\alpha]_D^{25}$ + 14° (C, 5%). There is no characteristic absorption of ultraviolet light. Titration with sodium hydroxide reveals two titratable groups with pKa values of 7.0 and 9.0 to 9.1 which become 5.0 and 7.5 in 4% formaldehyde solution. The X-ray diffraction pattern indicates that the dihydrochloride pentahydrate is completely crystalline. The infrared spectrum, run as a mineral oil mull, shows strong bands at 2.9 to 3.4, 10.79, and 11.58 $\mu$, bands of moderate intensity at 5.95 to 6.15, 6.36, 8.73, 9.01, 9.2, 9.29, 9.54, 9.63, 9.72, 9.97, 10.45, and 13.73 $\mu$, and weak bands at 7.39, 7.51, 8.49, 8.90, 10.62, 11.16, 11.39, and 12.21 $\mu$ (Fig. 1).

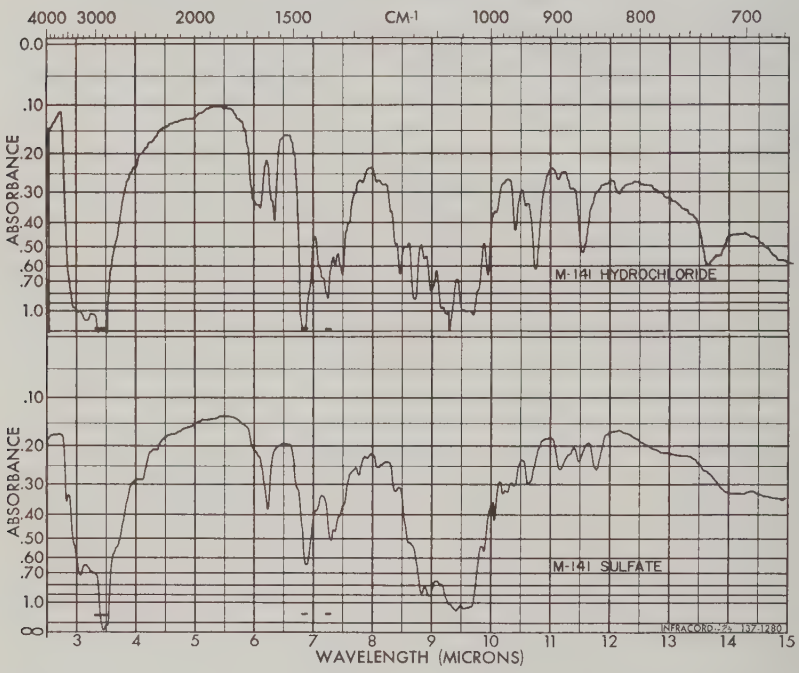


FIG. 1. *Infrared absorption spectrum of M-141 hydrochloride hydrate and M-141 sulfate hydrate in mineral oil suspension.*

The sulfate salt, recrystallized from acetone-water, is obtained as thin flat rectangular plates which decompose above 190 C. Elemental anlysis agrees with the formula $C_{14}H_{26\text{-}28}O_7N_2 \cdot H_2SO_4$ with

3 or 3.5 moles of water of crystallization. The X-ray diffraction pattern shows the material to be completely crystalline.

A sample of actinospectacin sulfate (Mason, Dietz, and Smith, 1961) was kindly supplied by G. M. Savage of the Upjohn Company, Kalamazoo, Mich. The M-141 sulfate and the actinospectacin sulfate give identical infrared spectra in mineral oil, and their bioautographs after paper chromatography in a variety of solvent systems are also identical. The bioautographs against *Bacillus subtilis* show a zone of complete inhibition next to the paper strip, a narrow band of stimulated growth around the zone of complete inhibition, a wide band of partial inhibition, and a second narrow band of stimulation. Paper strips (Eaton Dikeman 613) were developed by the upflow technique at 28 C. Some of the solvent systems used for developing the paper strips and the corresponding $R_F$ values are as follows: (i) *n*-butanol saturated with water, 0.03; (ii) *n*-butanol with 2% (w/v) *p*-toluenesulfonic acid, 0.32; (iii) *n*-butanol with 2% (v/v) pipiridine, 0.36; the bioautograph zone is smaller than in (i) and (ii); (iv) methanol-water (4:1) with 1.5% (w/v) sodium chloride; paper buffered with 1 M sodium sulfate-bisulfate (pH 2.0), 0.87; (v) same as (iv) but with ethanol instead of methanol, 0.60; (vi) water, 0.12 (streaming); (vii) water, 0.5% ammonium chloride, 0.79; and (viii) water, 20% ammonium chloride, 0.98.

The free base has been prepared from the sulfate salt by addition of the stoichiometric amount of barium hydroxide, but we have not succeeded in crystallizing it and doubt its purity. There is no detectable reduction in antibacterial activity in vitro or in vivo, but the elemental analysis is somewhat unsatisfactory, and the specific rotation is negative $\left[\alpha\right]_D^{25} = -11^o$ (C, 2.8%, water), although a solution of the hydrochloride salt made alkaline with sodium hydroxide and quickly transferred to a polarimeter tube shows a small positive rotation. Within 2 or 3 min, however, the rotation becomes negative. After 2 days, a fairly constant value is reached $\left[\alpha\right]_D^{25} = -43^o$ (C, 15%, 0.1 N NaOH). The infrared spectrum of the free base shows a carbonyl band at about 5.8 $\mu$ of such low intensity that it must be largely tied up, perhaps as a hemiacetal, but we have not investigated this point.

The hydrochloride salt was dissolved in water and subjected to low pressure hydrogenation over platinum oxide; 1 mole of hydrogen was taken up. The product crystallized with difficulty from acetone-water in long thin rectangular plates which darken at 185 C and melt with decomposition at about 208 C. The specific rotation is $\left[\alpha\right]_D^{25} = +28^o$ (C, 15%, water). When dissolved in water and made 0.1 N with sodium hydroxide, the specific rotation is $+22^o$ and does not change in 1 hr.

Dihydro M-141 has about one-tenth of the antibacterial activity in vitro of the natural antibiotic.

When the hydrochloride salt is subjected to acid hydrolysis, refluxing for 1 hr in 6 N hydrochloric acid, some carbon dioxide is evolved and the solution becomes dark brown. The only product we have isolated is a white crystalline salt, $C_8H_{16-18}O_4N_2 \cdot 2HCl$. This salt decomposes above 280 C on heating. It shows no optical rotation at the sodium D line. On titration with sodium hydroxide, pKa values of 7.2 and 9.1 are obtained. These are very close to the values for the antibiotic hydrochloride. The infrared spectrum shows bands attributable to OH or NH or both and secondary amine hydrochloride. The same compound is isolated after hydrolysis of the hydrogenated M-141 hydrochloride.

## Acknowledgments

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## Literature Cited

Mason, D. J., A. Dietz, and R. M. Smith. 1961. Actinospectacin, a new antibiotic. I. Discovery and biological properties. Antibiotics & Chemotherapy 11;118-122.

Oliver, T. J., A. Goldstein, R. R. Bower, J. C. Holper, and R. H. Otto. 1962. M-141, a new antibiotic. I. Antimicrobial properties, identity with actinospectacin, and production by *Streptomyces flavopersicus*, sp. n. Antimicrobial Agents and Chemotherapy - 1961, p. 495-502.

# M-141, A NEW ANTIBIOTIC
## III. Pharmacology

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A new wide-spectrum antibiotic, M-141, has been isolated from the fermentation beers of *Streptomyces flavopersicus*, sp. n. (Oliver et al., 1962). It has been prepared as a crystalline pentahydrate hydrochloride salt with the empirical formula: $C_{14}H_{26}O_8N_2 \cdot 2HCl \cdot 5H_2O$ (Sinclair and Winfield, 1962). According to these authors, M-141 is identical with actinospectacin produced by *S. spectabilis* NRRL 2792 as reported by Mason, Dietz, and Smith (1961).

M-141 has shown activities both in vitro and in vivo against a variety of gram-positive and gram-negative bacteria. Its efficacy in vivo in the protection of mice against various bacterial infections exceeded expectations from the activities in vitro (Oliver et al., 1962). These findings appear to be in accord with the results obtained with actinospectacin in a different laboratory (Mason et al., 1961; Lewis and Clapp, 1961). These promising properties of this new antibiotic prompted the present pharmacological investigation.

### Materials and Methods

*Assay procedure.* The M-141 preparation employed in this study was a crystalline pentahydrate hydrochloride salt (Sinclair et al., 1962) with an estimated purity of about 90% or better. All numerical values in this paper regarding this antibiotic are given on a weight basis of this preparation.

An agar diffusion zone assay with Labline steel plate was used for all M-141 activity determinations. An *Escherichia coli* strain (ATCC 26) was used as the test organism. The lower limits of the assay of the activities in the heparinized plasma, urine, and hepatic bile were respectively 5, 12.5 (at 1:50 dilution of the urine), and 20 $\mu$g/ml.

Most of the methods employed in the following experiments are similar to those reported previously (Hwang and Primack, 1957).

*Acute toxicity and tolerance.* Effects of single large doses of M-141 in aqueous solution were studied in various species of laboratory

animals (180 mice, 10 dogs, 8 cats, 4 monkeys, and 12 rabbits) by routes of administration.

*Subacute toxicity.* Preliminary studies were made in two male dogs receiving single daily oral doses of 50 mg/kg for 5 weeks followed by 500 mg/kg for 4 additional weeks. In 36 rats with an equal number of each sex, M-141 was given daily (500 and 1,000 mg/kg orally or intraperitoneally), with appropriate controls. Routine laboratory tests were carried out before, during, and at the end of the treatment. These included blood counts, hemoglobin determinations, hematocrit, and urinalysis in both species, and also blood chemistry (bilirubin, sugar, NPN, Na, K, Cl), liver function (bromsulphalein test), and renal function (phenolsulfonphthalein test) in the dog. All animals were observed for body weight changes and toxic signs. At the end of the experimental period, all animals were killed for histopathological studies.

*Pharmacodynamics.* Effects of various intravenous doses of M-141 were studied in dogs and cats anesthetized with pentobarbital. Blood pressure, respiration, and electrocardiogram were recorded with a Grass polygraph; in addition, the duodenal motility in the dog was recorded through a water-filled balloon transducer system.

*Absorption, distribution, excretion, and plasma level studies.* Plasma levels of M-141 activities and urinary excretion following single intravenous, intramuscular, and oral doses were studied in trained female dogs. These were also studied in pentobarbital-anesthetized dogs in which urine was collected from both ureters and hepatic bile from common bile duct after the cystic duct was clamped. In addition, aqueous humor and cerebrospinal fluid were also aspirated for assay. In two dogs under anesthesia, bilateral nephrectomy was performed before the blood level and hepatic excretion studies of M-141 were made. Plasma levels of M-141 activities were also studied in the monkey after oral doses. All dogs and monkeys were fasted for 18 hr before the oral administration of M-141.

*Mode of renal excretion.* The mode of renal excretion of M-141 was examined by renal clearance studies in four female dogs under pentobarbital anesthesia. The dogs were first hydrated, and then primed and infused at constant rate for sustaining blood levels with solutions containing appropriate amounts of M-141 and creatinine. The clearance ratios of these two substances were obtained from their simultaneous renal clearances in four to five consecutive periods of 10 min each.

*Protein binding properties.* The question whether M-141 is bound to any degree to the plasma proteins was examined by a dialysis technique (Tomsett, Schultz, and McDermott, 1947).

*Tissue irritation at the site of injection.* Solutions of M-141 (0.5 ml) of various concentrations were tested for tissue irritation by intramuscular (lumbar) administration to rabbits. The animals were killed 72 hr later, for examination of gross lesions at the site of injection.

*Potential antigenicity.* Possible antigenic effect of M-141 was tested in the guinea pig. Intramuscular doses of 200 and 500 mg/kg were each given to 10 pigs (6 doses, 2 to 3 days apart for 2 weeks). Intravenous challenging doses of 200 mg/kg were given 3 weeks after the last sensitizing dose.

## Results and Discussion

*Acute toxicity and tolerance.* The earlier results in the acute toxicity studies with female mice by the intravenous route indicated a low $LD_{50}$ of 2.0 g/kg. Doses of 1.25 g/kg or over were followed by transient depression and ataxia. All the deaths were preceded by coma and respiratory failure following acute depression and ataxia. There were no delayed deaths during an observation period of 1 week and no gross or histological lesions of the important organs when killed and autopsied. Later studies were made with a more uniformly controlled rate of injection at about 1 g per kg per 100 sec. No death occurred after a dose of 2.0 g/kg. The $LD_{50}$ was about 2.85 g/kg in the male and about 2.5 g/kg in the female. Signs at the lethal doses were similar to those described above.

Oral doses were tolerated by the mouse up to 10 g/kg. Only some loose stool was observed at these doses. Doses of 20 g/kg were followed by 35% deaths (14 mice). Autopsy revealed that the alimentary tract was distended with liquid contents apparently owing to poor absorption of the injected material. M-141 also showed low toxicity in the other species. In the dog, single oral doses up to 1 g/kg were tolerated without toxic signs. Intravenous doses were well tolerated up to 800 mg/kg. A dose of 1 g/kg was followed by emesis in one of the two dogs tested. In another dog, a dose of 2 g/kg caused emesis, ataxia, and transient collapse. In the cat, single intravenous doses of 400 mg/kg were well tolerated but a dose of 800 mg/kg was followed by a transient period of ataxia. Monkeys tolerated oral doses of 100 or 500 mg/kg without apparent effects.

Single intracisternal doses of M-141 to unanesthetized rabbits showed no toxic effects at 5 mg/kg and below. By the same route, doses of 10 and 20 mg/kg caused depression with occasional excitation and ataxia. After a dose of 30 mg/kg a rabbit had several clonic seizures followed by depression and ataxia for a period of 5 days before complete recovery. In another rabbit a dose of 40 mg/kg was followed by acute depression and respiratory failure.

*Subacute toxicity.* All rats and dogs showed no signs of toxicity from the M-141 administration. Laboratory and histological studies revealed no significant deviation from control animals.

*Pharmacodynamics.* In pentobarbital-anesthetized dogs or cats there were no significant changes following intravenous doses of 20 and 50 mg/kg of M-141. Higher doses were followed by transient

decrease of blood pressure, increased respiratory rate, bradycardia, and slight decrease of intestinal tone and motility. After doses of 400 to 800 mg/kg, the blood pressure and intestinal effects were more marked. Recovery took place gradually in about 20 to 35 min. These effects were not abolished nor prevented by bilateral vagotomy. During the acute depressor phase, the carotid sinus reflex was completely blocked and response to 1,1-dimethyl-4-phenylpiperazinium iodide (a ganglionic stimulating agent described by Chen, 1951) was diminished or abolished. Response to a standard dose of epinephrine was little altered from that obtained in the control phase. These findings suggested a blockade of the sympathetic system at the ganglionic level, except for the absence of the epinephrine potentiation. When intravenous doses of M-141 at 800 to 1,000 mg/kg were given to unanesthetized cats or dogs, there were no changes of the pupillary size or reflexes nor prolapse of the nictitating membranes. The apparent ganglionic blocking effect of M-141 at high doses in the anesthetized animals thus appears to be related somehow to the anesthetic used. This point is being further investigated.

Isolated rabbit ileum in aerated Tyrode solution kept at 37 C was treated with M-141 from 0.05 to 0.5 mg/ml in the bath. There were no changes in spontaneous activities nor in the responses to acetylcholine, barium chloride, histamine, or nicotine.

*Absorption, distribution, and excretion.* Plasma levels of M-141 activity following single intravenous or intramuscular doses in the unanesthetized dogs were essentially comparable in pattern (Fig. 1).

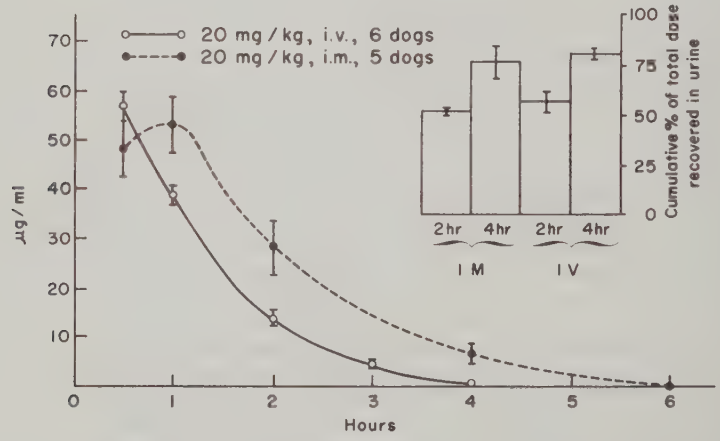


FIG. 1. *Plasma levels and renal excretion (inset) of M-141 in unanesthetized dogs after single intravenous (iv) or intramuscular (im) doses at 0 hr. Vertical bars represent standard errors of the means.*

The values at the 0.5- and 1-hr points were not significantly different by the Student's *t* test. But the differences at the second and fourth hours were significant, indicating a more sustained plasma level after intramuscular administration of this antibiotic. The rapid decline of the plasma concentration of M-141 activities corresponded to the prompt urinary excretion as shown in the inset of Fig. 1. About 80% (range 60 to 90%) of the total dose of M-141 given by either route was recovered in the urine in a course of 4 hr.

The absorption studies of M-141 after oral administration in the dog and monkey are shown in Table 1. Only relatively low plasma levels were attained after large doses in both species, particularly in the monkey. The mean urinary recovery of the M-141 activity in the dog was 9.3% of the total dose over a period of 6 hr.

TABLE 1. *Absorption of M-141 after single oral doses in the dog and monkey*

| | | Plasma level (μg/ml)* | | | | | Urinary recovery (cumulative dose) | | |
|---|---|---|---|---|---|---|---|---|---|
| Animal | Dose | 0.5 hr | 1 hr | 2 hr | 4 hr | 6 hr | 2 hr | 4 hr | 6 hr |
| *Dog* | *mg/kg* | | | | | | % | % | % |
| 1 | 500 | 11.5 | 38 | 57 | 46 | 28 | 2.34 | 5.74 | 7.88 |
| 2 | 500 | 5 | 5 | 10.5 | 16.5 | 11 | .30 | .79 | 1.14 |
| 1 | 100 | 5.1 | 9.4 | 7.6 | 4 | 4 | 1.90 | 3.95 | 4.89 |
| 2 | 100 | 0 | 0 | 8.4 | 4.9 | 4 | 1.69 | 3.98 | 5.66 |
| 3 | 100 | 0 | 0 | 14.5 | 7.0 | 0 | 1.75 | 6.20 | 7.60 |
| 4 | 100 | 0 | 0 | 11.5 | 10.0 | 4 | 2.26 | 6.56 | 9.33 |
| 5 | 100 | 6.2 | 16.7 | 17.5 | 15.7 | 9.5 | 1.81 | 8.39 | 16.90 |
| 6 | 100 | 4 | 10.5 | 16.9 | 8.9 | 5.2 | 3.13 | 5.44 | 8.37 |
| 7 | 100 | 4 | 7.1 | 15.0 | 11.4 | 5.5 | 2.36 | 6.53 | 9.07 |
| 8 | 100 | 4 | 6.0 | 6.7 | 8.3 | 5.1 | 3.14 | 6.00 | 8.92 |
| 9 | 100 | 16.6 | 28.5 | 19.7 | 6.1 | 4 | 8.71 | 12.71 | 15.10 |
| 10 | 100 | 4.7 | 12.5 | 17.7 | 8.6 | 7.2 | 2.77 | 5.13 | 6.93 |
| Mean ± standard error | | 4.46± 1.53 | 9.07± 2.81 | 13.6± 1.48 | 8.49± 1.08 | 4.85± 0.76 | 2.97± .66 | 6.49± .66 | 9.28± 1.22 |
| *Monkey* | | | | | | | | | |
| 1 | 500 | | | 5.4 | 10.0 | | | | |
| 2 | 500 | | | 0 | 4 | | | | |
| 3 | 100 | | | 6.6 | 5.2 | | | | |
| 4 | 100 | | | 0 | 4 | | | | |

*M-141 activities less than 5 μg/ml are approximate values.

In dogs under pentobarbital anesthesia the plasma levels after single intravenous doses of M-141 (Table 2) were similar to the curve as seen in the unanesthetized dogs (Fig. 1). The urinary recovery was seemingly complete in 4 to 5 hr. The urinary concentration in all dogs after either intravenous or intramuscular doses varied with the rate of urine flow, but often exceeded levels of 10,000 μg/ml. On the other hand, the hepatic bile levels were very low, usually much less than the mean plasma value of the corresponding period. The amount of M-141 activity excreted through the bile

TABLE 2. *Hourly plasma levels, distribution, and excretion of M-141 single intravenous doses in pentobarbital anesthetized dogs with and without kidneys**

| Dose | Plasma (μg/ml) | | | | | | | Urinary recovery (cumulative total dose) | | | | | | Hepatic bile, μg/ml (volume) | | | | | Aqueous humor (μg/ml) | | Cerebrospinal fluid (μg/ml) | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 0.25 | 0.5 | 1 | 2 | 3 | 4 | 5 | 0.5 | 1 | 2 | 3 | 4 | 5 | 1 | 2 | 3 | 4 | 5 | 1 | 2 | 1 | 3 |
| *mg/kg Kidneys intact* | | | | | | | | % | % | % | % | % | % | | | | | | | | | |
| 40 | 192 | 133 | 102 | 46.5 | 27 | 18.2 | 11.5 | | 43.2 | 69.3 | 82.8 | 93.2 | 103.5 | 38.4 | 56 | 30.4 (2.4-2.7 ml/hr) | 17.6 | 5.5 | 9 | 4.2 | 0 | |
| 20 | 85 | 50.5 | 32 | 13 | 5.4 | 4 | | 55.9 | 69.4 | 90.5 | 96.7 | 104 | | 16 | 14 | 0 (5.0-10.3 ml/hr) | 0 | | | | 0 | |
| 20 | 83.5 | 57 | 38.3 | 14.3 | 6.2 | 4 | | 30.2 | 56.5 | 80.8 | 92.5 | 97.4 | | 15.6 | 13 | 10 (2.9-3.0 ml/hr) | 0 | | 9 | 8 | | |
| *Kidneys removed* | | | | | | | | | | | | | | | | | | | | | | |
| 15 | 83 | 74 | 67 | 60 | 59 | 50 | | | | | | | | 37.5 | 30 | 30 (4.5-8.9 ml/hr) | | | 9 | 0 | 0 | |
| 15 | | 101 | 89.5 | 87 | 82.5 | 76 | 76.5 | | | | | | | 47.2 | 42.2 | 42.7 | 42.5 | | 5 | 9 | | 0 |
| | | | | | | | | | | | | | | | | (1.2-2.3 ml/hr) | | | | | | |

*M-141 activities less than 5 μg/ml in the plasma or less than 20 μg/ml in the other fluids are approximate values.

was practically negligible under these experimental conditions. Only a trace of activity was found in the aqueous humor and none was found in the cerebrospinal fluid at the height of the plasma levels.

The importance of the renal excretion was further borne out in two bilaterally nephrectomized dogs (Table 2). The plasma activities of M-141 after a single intravenous dose were sustained at high levels for several hours. The hepatic bile activities were also sustained but only at a level of about one-half of the plasma values. However, the M-141 activities were still very low in aqueous humor and nil in cerebrospinal fluid.

*Mode of renal excretion and protein-binding properties.* Renal clearance of M-141 in the dog was close or identical to that of the simultaneous clearance of creatinine (Table 3). The mean clearance ratio of M-141 to creatinine was 0.95 (± standard error 0.032).

TABLE 3. *Simultaneous renal clearances of M-141 and creatinine in four female dogs**

| Wt | Rate of urine flow | M-141 | | | Creatinine | | Clearance ratio (M-141/ creatinine) |
|---|---|---|---|---|---|---|---|
| | | Urinary concen | Mean plasma level | Clearance† | Mean plasma level | Clearance† | |
| *kg* | *ml/min* | *μg/ml* | *μg/ml* | | *μg/ml* | | |
| 11.8 | 0.95 | 5,158 | 150 | 2.77 | 107 | 3.25 | 0.85 |
| | 1.10 | 4,633 | 165 | 2.62 | 105 | 2.97 | 0.88 |
| | 1.05 | 5,586 | 160 | 3.11 | 106 | 2.82 | 1.10 |
| | 1.05 | 5,197 | 175 | 2.64 | 104 | 2.74 | 0.97 |
| Mean | | | 162.5 | 2.785 | 105.5 | 2.945 | 0.95 |
| 9.4 | 0.60 | 7,210 | 140 | 3.29 | 87.7 | 4.00 | 0.83 |
| | 0.65 | 6,748 | 140 | 3.33 | 82.5 | 3.77 | 0.89 |
| | 0.60 | 8,190 | 143 | 3.69 | 82.5 | 4.24 | 0.84 |
| | 0.65 | 8,071 | 153 | 3.65 | 84.0 | 4.05 | 0.90 |
| Mean | | | 144 | 3.490 | 84.18 | 4.015 | 0.87 |
| 8.9 | 2.80 | 1,875 | 158 | 3.53 | 72.0 | 4.13 | 0.85 |
| | 2.70 | 1,965 | 145 | 4.15 | 73.5 | 4.03 | 1.03 |
| | 2.73 | 2,085 | 169 | 3.79 | 74.3 | 3.92 | 0.97 |
| | 2.65 | 1,995 | 157 | 3.80 | 74.3 | 3.75 | 1.01 |
| | 2.95 | 1,950 | 171 | 3.80 | 76.5 | 4.10 | 0.93 |
| Mean | | | 160 | 3.814 | 74.12 | 3.986 | 0.96 |
| 9.5 | 3.30 | 1,725 | 143 | 4.16 | 89.4 | 3.78 | 1.10 |
| | 2.60 | 1,905 | 137 | 3.79 | 85.5 | 3.48 | 1.09 |
| | 2.10 | 2,070 | 158 | 2.88 | 87.8 | 2.95 | 0.98 |
| | 2.25 | 2,025 | 167 | 2.86 | 88.5 | 2.92 | 0.98 |
| | 2.25 | 1,965 | 173 | 2.67 | 89.4 | 2.67 | 1.00 |
| Mean | | | 155.6 | 3.272 | 88.12 | 3.160 | 1.03 |
| Group mean ± standard error | | | | 3.34 ± 0.21 | | 3.53±0.26 | 0.95 ± 0.032 |

*Sustaining intravenous infusion was given after priming dose during the experiment under pentobarbital anethesia. Mannitol (5%) was included in the sustaining fluid in the last two dogs. Each consecutive period of clearance was 10 min in duration.

†Expressed as ml per min per kg.

This would suggest that M-141, similar to creatinine, was excreted by glomerular filtration without significant participation of the tubular function if the antibiotic was not bound to the plasma proteins. This was apparently the case as shown by the dialysis experiment (Table 4). In this experiment, M-141 in dog plasma (90%) was dialyzed against saline with a gradient ratio of 10:1. The blank plasma (90%) and saline bags served as controls to indicate the equilibrium end points. Apparently M-141 diffused rather readily through the membranes and reached equilibrium in about 4 to 6 hr. No protein binding of M-141 was revealed.

TABLE 4. *Protein binding studies with M-141**

| Dialysate | Before dialysis | After dialysis (μg/ml) | | | |
|---|---|---|---|---|---|
| | | 2 hr | 4 hr | 6 hr | 29 hr† |
| | *μg/ml* | | | | |
| Saline (75 ml) | 51.8 | 64.0 | 65.5 | 63.0 | 64.0 |
| Saline bag (3 ml) | 0 | 51.0 | 61.0 | 60.0 | 62.5 |
| Plasma bag 1 (3 ml) | 512 | 90.0 | 56.5 | 51.2 | 45.7 |
| Plasma bag 2 (3 ml) | 0 | 38.5 | 48.2 | 49.5 | 51.2 |
| Total activity (μg) | 5421.0 | 5338.5 | 5409.6 | 5207.1 | 5278.0 |

*Plain saline and dog plasma (90%) with or without added M-141 were placed in separate cellophane bags and shaken in saline with a known M-141 concentration at room temperature.

†Only 10 hr of shaking at room temperature. During the remaining time the system was kept at 5 C.

*Tissue irritation by intramuscular route.* Intramuscular injections of M-141 solution of various concentrations were well tolerated by the rabbit. Slight to moderate tissue necrosis was present at the site of injection of 30 to 40% solutions of M-141 when the rabbit was killed and examined 3 days postinjection. However, when a 20% solution of M-141 was used there were no signs of irritation.

*Potential antigenicity studies.* In view of the rapid and complete absorption without apparent tissue irritation by intramuscular administration of a 20% solution of M-141, the antigenicity test in the guinea pigs was carried out by sensitizing the pigs by giving M-141 (as a 20% solution) by this route. All pigs received the challenging dose of M-141 intravenously. None showed any untoward signs suggesting hypersensitivity.

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## Literature Cited

Chen, G., R. Portman, and A. Wickel. 1951. Pharmacology of 1, 1-dimethyl-4-phenyl-piperazinium iodide, a ganglion stimulating agent. J. Pharmacol. Exptl. Therap. 103:330-336.

Hwang, K., and N. Primack. 1957. Pharmacologic studies of Synnematin B. Antibiotics Ann. 1956-1957, p. 755-780.

Lewis, C., and H. W. Clapp. 1961. Actinospectacin, a new antibiotic. III. In vitro and in vivo evaluation. Antibiotics & Chemotherapy 11:127-133.

Mason, D. J., A. Dietz, and R. M. Smith. 1961. Actinospectacin, a new antibiotic. I. Discovery and biological properties. Antibiotics & Chemotherapy 11:118-122.

Oliver, T. J., A. Goldstein, R. R. Bower, J. C. Holper, and R. H. Otto. 1962. M-141, a new antibiotic. I. Antimicrobial properties, identity with actinospectacin, and production by *Streptomyces flavopersicus*, sp. n. Antimicrobial Agents and Chemotherapy - 1961, p. 495-502.

Sinclair, A. C., and A. F. Winfield. 1962. M-141, a new antibiotic. II. Isolation and characterization and identity with actinospectacin. Antimicrobial Agents and Chemotherapy-1961, p. 503-506.

Tompsett, R., G. Schultz, and W. McDermott. 1947. The relation of protein binding to the pharmacology and antibacterial activity of penicillins S, G, dihydro F, and K. J. Bacteriol. 53: 581-595.

# IN VIVO AND IN VITRO DEVELOPMENT OF RESISTANCE AGAINST ACTINOSPECTACIN

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The availability of actinospectacin (Mason, Dietz, and Smith, 1961) has been the source of some encouragement for its use as an effective agent against the common gram-negative pathogens. In comparison to some of the other antibiotics, actinospectacin appears to offer no pronounced advantages over such antibiotics as penicillin, erythromycin, or chloramphenicol with staphylococci. The poor correlation between sensitivity test results and the in vivo activity of actinospectacin therapy seems to be paralleled by poor correlation between disc sensitivity results and minimal inhibitory concentrations (MIC), conducted in the usual manner (Sokolski, Chidester, and Schadewald, 1961).

## Materials and Methods

The in vivo activity of actinospectacin was studied in experimental infections in rabbits and mice. For the first part of the evaluation, 12 albino rabbits (3,000 g each) were used. Of these, four rabbits were given 1 ml of an 18-hr brain heart infusion broth culture of *Staphylococcus aureus*. This staphylococcus was a recently isolated strain with a phage type 52,52A,80,81. This strain was resistant to actinospectacin, tetracycline, and penicillin, slightly sensitive to novobiocin, and sensitive to chloramphenicol. The second group of four animals received the same amount of brain heart infusion broth culture of a recently isolated strain of *Proteus vulgaris*, and the last group received 1 ml of a brain heart infusion broth culture of *Escherichia coli*. The *Proteus* strain was very sensitive to actinospectacin and chloramphenicol and slightly sensitive to novobiocin. The *E. coli* strain was also sensitive to actinospectacin and chloramphenicol and was completely resistant to novobiocin. All rabbits received their injections intravenously. One animal in each group was left as an untreated control.

In the mouse series, three groups of 10 mice each were injected by the intraperitoneal route with approximately 200 million cells of

*P. vulgaris*, *E. coli*, or *Pseudomonas aeruginosa*. All the mice used in this experiment weighed 25 g or more.

In all cases, the animals received treatment immediately after being injected with the bacterial cells. The treatment in both series of animals was continued for 5 days and was divided into three equal doses per day so that each animal received actinospectacin at a rate of 40 μg per kg per day.

In vitro sensitivity testing of 34 recently isolated strains of gram-negative bacilli was performed on streptomycin assay agar and peptone agar. The organisms tested included *Proteus*, *Escherichia*, *Pseudomonas*, *Aerobacter*, and *Klebsiella* species. The MIC tests were conducted in streptomycin assay broth adjusted to a pH of 7.9. The MIC's were set up at concentrations of 1, 4, 20, 50, and 100 μg/ml. These were read after 24 and 48 hr, and all concentrations were cultured at the end of 48 hr. Cultural isolations made from the 100-μg tube were retested in a further series of dilutions of 100, 250, and 500 μg/ml. Culturing of the 500-μg tube was performed to determine bactericidal or bacteriostatic effect of actinospectacin. In an effort to study the correlation between discs and tube dilution tests, as influenced by a difference in disc concentration, two series of tests were conducted. In the first series, discs of 2, 10, 40, 100, 150, 200, 250, 300, 350, and 400 μg each were compared against MIC results obtained from tubes containing 1, 4, 20, 50, and 100 μg/ml of streptomycin assay broth. In a second series, to define more closely the optimal disc concentration, discs of 10, 20, 30, 40, 50, 60, 70, 80, 90, 100, 125, and 150 μg each were studied against MIC's of 10, 20, 30, 40, 50, 60, 70, 80, 90, and 100 μg/ml of streptomycin assay broth.

## Results

All controls in the rabbit series were dead either the first or second day. In the treated series of rabbits receiving staphylococci, one rabbit lived for 5 days. In the series receiving *E. coli*, one rabbit was cured at the end of 5 days, and it was impossible to culture *Escherichia* from this animal at the time of autopsy. In the *Proteus* series, two animals were cured at the end of 5 days, and it was impossible to culture *Proteus* from these animals at autopsy. Blood cultures taken from the marginal vein of the ear on the fourth day of therapy were also found to be negative. Positive blood cultural results were obtained from one rabbit of the staphylococcus series. Positive cultures were obtained from the spleen, kidney, and liver of all rabbits of the *Staphylococcus* series when these tissues were examined at autopsy. Repeated sensitivity testing of the *Staphylococcus*, after animal passage and 5 days contact with actinospectacin, showed no differences in the sensitivity results.

In the mouse series, none of the injected animals died. The day after completion of therapy, these animals were autopsied and cultures made from peritoneal washings, spleen, liver, and lungs. The only organisms recovered were from the peritoneal cavities of the mice in the *P. vulgaris* group. When these isolations were tested for sensitivity, both by the tube and disc methods, they showed reactions identical to ones before animal passage and treatment.

In vitro sensitivity testing of stock strains of gram-negative organisms, with actinospectacin discs on streptomycin assay agar and peptone agar, disclosed that differences in sensitivity could be observed on different media. A total of 34 recently isolated gram-negative organisms was tested in this manner. MIC tests conducted in streptomycin assay broth, adjusted to a pH of 7.9, showed that most of the gram-negative bacilli were sensitive to 1 $\mu$g/ml, with the exception of *Pseudomonas,* which was uniformly resistant to an excess of 20 $\mu$g of actinospectacin per ml. With the exception of one strain of *Proteus* and two of *Escherichia,* the only organisms showing resistance above 100 $\mu$g were the *Pseudomonas* strains. These grew out at 500 $\mu$g/ml. After 48 hr incubation, cultures made from the highest concentration, the 500-$\mu$g tube, produced growth in all but one strain of *Escherichia,* showing conclusively that actinospectacin is more bacteriostatic than bactericidal.

The limited series of gram-negative organisms tested indicate that extreme caution should be used in interpreting the disc sensitivities in the light of comparable tube dilution tests. These results do not seem to be parallel. The tube dilution tests with gram-negative organisms indicate that, with the exception of *Pseudomonas,* most organisms are sensitive to concentrations in the neighborhood of 1 $\mu$g/ml. In the limited animal series so far studied, this seems to apply, since it was impossible to recover gram-negative organisms from the rabbit series at autopsy and only the *Proteus* strain was recovered from the mouse series.

When a correlation was made between the results of the MIC's and the concentration of the discs producing at least 10–mm zones of inhibition, it was found that a figure of 60 $\mu$g/disc was obtained in the first experiment, and, when the experiment was repeated, using narrower values for the MIC's and discs, a value of 45 $\mu$g/disc showed complete correlation with the MIC.

## Discussion

In the limited animal series, actinospectacin displayed effective antibacterial activity against the gram-negative organisms studied. The same degree of activity was not demonstrated against the series of animals infected with staphylococci. Although actinospectacin may not be of as high an order of reactivity against the gram-negative

organisms as some of the now available antibiotics, it may still be a valuable therapeutic adjunct since cross-resistance to actinospectacin was not demonstrated by organisms resistance to other drugs. The organisms reisolated from treated animals did not show a change in sensitivity after animal passage and 5 days of contact with the antibiotic. The development of in vivo resistance may be slower than in vitro. Although most of the gram-negative organisms were sensitive to 1 μg/ml of actinospectacin, universal resistance of some degree was shown by *Pseudomonas* species and occasionally by other genera. The *Pseudomonas* strains growing in 500 μg/ml of actinospectacin gave good growth after 48 hr, indicating a bacteriostatic action rather than a bactericidal action of the drug. The apparent influence of media on sensitivity results is a distinct disadvantage if discs are to be used. It is impossible for the average laboratory to reserve a special medium for actinospectacin alone. Elucidation of these points, concerning medium and higher concentration discs, showed that effective and usable discs can be made at a concentration somewhere between 45 and 60 μg/disc. If discs of this concentration are employed with tryptose agar plus 5% added blood, good correlation can be obtained between the MIC and the disc method.

## Literature Cited

Mason, D. J., A. Dietz, and R. M. Smith. 1961. Actinospectacin, a new antibiotic. Antibiotics & Chemotherapy. 11:118-122.

Sokolski, W. T., C. G. Chidester, and L. J. Schadewald. 1961. Human whole blood as a medium for testing inhibition by antibacterial substances. Bacteriol. Proc., p. 63.

# SUSCEPTIBILITY OF STAPHYLOCOCCI TO ACTINOSPECTACIN

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Actinospectacin is a new broad-spectrum antibiotic isolated from broth cultures of *Streptomyces spectabilis.* The biological properties, microbiological assays, and in vivo and in vitro activities of actinospectacin were reported recently in a series of companion papers (Mason, Dietz, and Smith, 1961; Hanka, Mason, and Sokolski, 1961; Lewis and Clapp, 1961). This report describes the comparative susceptibility of clinical isolates of *Staphylococcus aureus* to actinospectacin and 12 other antibiotics, and the minimal inhibitory concentration of actinospectacin using agar-dilution, broth-dilution, and disc-susceptibility techniques.

## Materials and Methods

*Selection of microorganisms.* The staphylococci tested in this study were originally isolated by Richard R. Menard from patients seen in this department. Only *S. aureus,* one strain from each patient and this from a lesion, was included. Of the 366 strains studied, 15% were isolated in 1959, 54% in 1960, and 31% in 1961. They had a variety of clinical sources, antibiograms, and phage types, as previously reported (Greer, Menard, and Livingood, 1961). Each stock culture was checked for purity by confirming the antibiogram number (Greer, 1959*a*, *b*). *S. aureus* 209P (ATCC 6538P) was also tested by all three methods, agar dilution, broth dilution, and disc susceptibility.

*Antibiotics.* Actinospectacin sulfate standard powder and 30- and 300-$\mu$g discs were obtained from The Upjohn Co., Kalamazoo, Mich. All discs used were SensiDiscs and supplied by Baltimore Biological Laboratory, Baltimore, Md. (BBL), except the 1- and 2-$\mu$g discs of 5-methyl-3-phenyl-4-isoxazolyl penicillin which were made and supplied by Bristol Laboratories, Inc., Syracuse, N.Y. The other 11 antibiotics used in discs were dihydrostreptomycin, penicillin G, phenethicillin, erythromycin, chloramphenicol, tetracycline, bacitracin, neomycin, kanamycin, vancomycin, and methicillin.

*Culture media.* Trypticase soy agar (BBL) was used for the actinospectacin susceptibility testing of all 366 strains by the agar dilution technique, and for the testing of 260 of these strains using 30- and 300-$\mu$g discs. It was also used for the stock cultures and disc susceptibility testing of all strains to the 11 other antibiotics. It was also the base used for blood agar plates made with 5% sterile, defibrinated sheep blood (Cappel Laboratory, West Chester, Pa.). Streptomycin assay agar with yeast extract (BBL) was used to test 136 strains with 30-$\mu$g actinospectacin discs and 10-$\mu$g dihydrostreptomycin discs. Trypticase soy broth (BBL) was used for the broth dilution tests.

*Techniques.* The susceptibility testing techniques followed the methods described by Grove and Randall (1955). In the agar-dilution tests, the replicator described by Steers, Foltz, and Graves (1959) was employed. The dilution of the inoculum for the broth-dilution tests was 1:1,000 of a 24-hr culture.

## Results

The minimal inhibitory concentrations of actinospectacin ranged from 50 to 100 $\mu$g/ml with susceptible strains and from 200 to more than 300 $\mu$g/ml with resistant strains by both agar-dilution and broth-dilution techniques using Trypticase soy agar. All strains grew at concentrations of 25 $\mu$g/ml, but there was more growth of "persistors" on streptomycin assay agar with yeast extract. Actinospectacin-susceptible strains were inhibited by both 30- and 300-$\mu$g discs on both agars. Zones of inhibition were larger on the streptomycin agar with yeast extract than on the other agar, but the difference was not sufficient to change the susceptibility classification. The addition of 5% sterile, defibrinated sheep blood did not alter the zone of inhibition around actinospectacin discs.

In 25 tests by all three methods using Trypticase soy agar or broth, the disc- and broth-dilution results agreed in 24, agar and broth dilution in 19, but all three in only 18. Only one strain was sensitive by disc tests but resistant by agar- and broth-dilution tests. Disc results never agreed with agar-dilution results without also agreeing with broth-dilution results. Of the entire 366 strains, 31 were considered sensitive by the disc test but resistant by agar dilution. Growth of *S. aureus* 209P, which was inhibited by 30-$\mu$g discs on both agars, occurred at actinospectacin concentrations of 25 but not at 50 $\mu$g/ml.

None of the 366 strains was resistant to vancomycin, methicillin, or 5-methyl-3-phenyl-4-isoxazolyl penicillin. Although 87 strains (24%) were resistant to the 10-$\mu$g dihydrostreptomycin disc on Trypticase soy agar, use of streptomycin assay agar did not result in

inhibition by this disc, but zones around actinospectacin discs were larger. There were 238 strains (65%) resistant to both penicillin G and phenethicillin, 36 (10%) resistant to erythromycin, two (1%) resistant to chloramphenicol, 108 (29%) resistant to tetracycline, four (1%) resistant to bacitracin, one (1%) resistant to both kanamycin and neomycin, and 39 (11%) resistant to actinospectacin. But 120 strains (33%) were not resistant to any of the 13 antibiotics.

The number of actionspectacin-resistant strains among the erythromycin-resistant ones (35 of 36) suggested a unilateral cross-resistance of these antibiotics. Since at one time in the study all erythromycin-resistant strains appeared actinospectacin-resistant, all subsequently isolated erythromycin-resistant strains were tested with actinospectacin. Four strains, in addition to the 366 mentioned above, are particularly interesting. One was isolated from an eczematous lesion over the right clavicle of a patient when hospitalized. This strain was sensitive to all 13 antibiotics. Erythromycin propionate lauryl sulfate was prescribed (250 mg, four times daily) for the next four days and discontinued on the fifth. On the sixth, eighth, and 13th days, strains were again isolated from the lesion but each of the three was now resistant to both erythromycin and actinospectacin, but sensitive to the other 11 antibiotics. None of these was lysed by any of the 24 phages listed previously (Greer et al., 1961) or by phages KS6 or 82. Nor were any shown to be lysogenic by cross-inoculation onto the propagating strains for these phages.

Although 36 of the 39 actinospectacin-resistant strains were erythromycin-resistant, cross-resistance with any single antibiotic or group of antibiotics was not observed. Actinospectacin-resistant strains, susceptible to the other 12 antibiotics, were also observed. Furthermore, various phage types were found among both actinospectacin-susceptible and actinospectacin-resistant strains.

## Discussion

Since both dilution tests were not performed on all strains in the study, and the disc tests results were closely correlated in the few done by all three methods, the total resistance of 11% is based on the disc results. If the 35 strains resistant by either or both of the dilution methods are counted as resistance, the incidence of actinospectacin resistance in this sample is approximately 20%. Although the agar-dilution method has been used satisfactorily in this laboratory for testing the susceptibility of staphylococci to other antibiotics, mostly penicillin, it is not the technique of choice for actinospectacin susceptibility testing. The end points are less distinct than with the other techniques. Neither the 30- nor the 300-$\mu$g disc seems superior for susceptibility testing. Even though the disc and broth techniques appear preferable because of the superior

correlation of results in this study, no clinical data are available supporting the validity of either result.

## Acknowledgments

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## Literature Cited

Greer, J. E. 1959*a*. Numerical designation of staphylococcal antibiograms. Antibiotics Ann. 1958-1959, p. 881-882.

Greer, J. E. 1959*b*. Addendum to the numerical designation of staphylococcal antibiograms. Am. J. Med. Technol. **25**:398.

Greer, J. E., R. R. Menard, and C. S. Livingood. 1961. Some bacteriological data on dermatological patients. I. Antibiograms and phage types of staphylococci. J. Invest. Dermatol. **36**: 273-282.

Grove, D. C., and W. A. Randall. 1955. Assay methods of antibiotics, a laboratory manual. Medical Encyclopedia, Inc., New York.

Hanka, L. J., D. J. Mason, and W. T. Sokolski. 1961. Actinospectacin, a new antibiotic. II. Microbiological assay. Antibiotics & Chemotherapy **11**:123-126.

Mason, D. J., A. Dietz, and R. M. Smith. 1961. Actinospectacin, a new antibiotic. I. Discovery and biological properties. Antibiotics & Chemotherapy **11**:118-122.

Lewis, C., and H. W. Clapp. 1961. Actinospectacin, a new antibiotic. III. In vitro and in vivo evaluation. Antibiotics & Chemotherapy **11**:127-133.

Steers, E., E. L. Foltz, and B. S. Graves. 1959. An inocula replicating apparatus for routine testing of bacterial susceptibility to antibiotics. Antibiotics & Chemotherapy **9**:307-311.

# LABORATORY AND CLINICAL STUDIES ON ACTINOSPECTACIN-M141, A NEW BROAD-SPECTRUM ANTIBIOTIC

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Actinospectacin is a new antibiotic isolated from *Streptomyces spectabilis* (Mason, Dietz, and Smith, 1961) and M141 is a new antibiotic isolated from *S. flavopersicus* (Oliver et al., 1962). These have been shown to be identical by Oliver et al. (1962), and by Sinclair and Winfield (1962). In this paper they will be referred to as a single antibiotic, actinospectacin-M141.

This is a report on laboratory evaluation still in progress of actinospectacin-M141 and its use in the treatment of 35 patients.

## Materials and Methods

Actinospectacin sulfate was supplied through the courtesy of The Upjohn Company, Kalamazoo, Mich., and M141 was supplied through the courtesy of Abbott Laboratories, North Chicago, Ill.

In vitro sensitivity tests were carried out by a tube dilution method; minimal inhibitory concentration (MIC) was recorded after 24 and 48 hr of incubation at 37 C. These tests were performed in brain heart infusion (Difco) at pH 7.4 (BHI 7.4) and in streptomycin assay broth at pH 7.9 (SAB 7.9). Bactericidal end points were determined by using 0.1 ml of dilutions showing no growth at the end of 48 hr as inoculum for pour plates.

Microbiological assay for the antibiotic was carried out by a serial dilution method using a strain of *Gaffkya tetragena* isolated in our laboratory which was sensitive to 1.0 $\mu$g/ml of actinospectacin-M141 in BHI 7.4. A 0.5-ml inoculum was made from a 24-hr culture of *G. tetragena* by diluting 1:1,000 to 5,000 and contained 10,000 to 20,000 microorganisms per ml. This microorganism was not inhibited by individual or pooled normal human sera.

Crossover studies were performed in noninfected subjects to test the comparative effectiveness of actinospectacin-M141 with that of chloramphenicol. Actinospectacin-M141 and chloramphenicol were alternately administered intramuscularly with a 2-day interval to three patients. Sera were then tested for antibacterial activity against a *Paracolobactrum* sp. isolated from a patient with sepsis.

Lewis and Clapp (1961) have reported that actinospectacin given orally is relatively ineffective in animals. Our studies have shown that, with currently available preparations, single oral doses up to 2.0 g of M141 give no detectable serum antibiotic activity. Therefore, actinospectacin-M141 was administered in therapeutic trials intramuscularly or intravenously in doses of 0.4 to 1.0 g (base equivalent) at intervals of 4 to 8 hr. For intravenous administration, the agent was diluted in 125 ml of 5% dextrose in water and administered in 15 to 20 min.

Tests of renal, hepatic, and hemopoietic function were performed before and at intervals during therapy.

## Results

Table 1 shows the in vitro sensitivity tests of actinospectacin-M141 against 88 strains of bacteria. All were recent isolates from pathologic sources. They were invariably more sensitive in streptomycin assay broth. Of the 59 gram-negative strains, 40 were inhibited in SAB by 10 μg/ml or less. Bactericidal effect was shown in 26 by 50 μg/ml or less. Of 29 gram-positive strains, 19 were inhibited by 10 μg/ml or less. Bactericidal effect was shown in 15

TABLE 1. *In vitro sensitivity of 88 bacterial strains to actinospectacin-M141*

| Microorganism | Range of sensitivity (μg/ml) | | | | Number tested | 24-Hr MIC* | | | Bactericidal* 50 μg or less |
|---|---|---|---|---|---|---|---|---|---|
| | BHI (pH 7.4) | | SAB (pH 7.9) | | | 5 μg or less | 10 μg or less | 50 μg or less | |
| | MIC | Bactericidal | MIC | Bactericidal | | | | | |
| *E. coli* | 10->50 | 10->50 | 1-3 | 2-5 (>50)† | 11 | 11 | | | 10 |
| *Paracolobactrum* sp. | 5->50 | 20->50 | 2-10 | 2->50 | 10 | 9 | 10 | | 5 |
| *Proteus* sp. | 30->50 | 30->50 | <1->50 | 3->50 | 12 | 7 | 8 | 9 | 6 |
| *K. pneumoniae* | 5->50 | 10->50 | 2->50 | 3->50 | 5 | 2 | | 4 | 2 |
| *A. aerogenes* | 10->50 | 20->50 | 2->50 | 4->50 | 11 | 4 | | 5 | 2 |
| *Pseudomonas* sp. | 30->50 | >50 | 3-40 | 30->50 | 8 | 1 | 3 | 8 | 1 |
| *Salmonella* sp. | 20-30 | >50 | 3 | >50 | 2 | 2 | | | 0 |
| *D. pneumoniae* | 5-30 | 5-30 | 1-2 | 2 | 5 | 5 | | | 5 |
| *S. mitis* | 10-30 | 20-30 | 3-4 | 10-30 | 3 | 3 | | | 3 |
| *S. pyogenes* | 20-30 | 20-30 | 3-5 | 3-30 | 5 | 5 | | | 5 |
| *S. aureus* | 20->50 | 40->50 | 3->50 | 20->50 | 11 | 2 | 4 | | 2 |
| Enterococcus | 30->50 | >50 | 2->50 | >50 | 5 | 2 | | 4 | 0 |
| Total | | | | | 88 | 53 | 59 | 70 | 41 |

*In SAB, pH 7.9.
†One strain bactericidal >50.

strains by 50 μg/ml or less. The difference between inhibitory and bactericidal concentrations was generally wide. The narrowest range was seen with pneumococci and *Escherichia coli*.

Lewis and Clapp (1961) noted that several strains of *Staphylococcus aureus* developed resistance rapidly in vitro to this antibiotic but that several strains of other microorganisms developed it more slowly. Our preliminary in vitro studies with one strain of *E. coli*, *Paracolobactrum* sp., and *Salmonella* sp. generally show a relatively slower development of resistance, which in some instances has been reversible after transfers without antibiotic. No evidence of cross-resistance to other antibiotics was noted.

The activity of actinospectacin-M141 in serum was related to dosage (Table 2). The serum level for a single dose of 1.0 g, 1 hr after intravenous administration in four subjects, was 20 to 40 μg/ml; for 1.0 g intramuscularly, 10 to 40 μg/ml; for 800 mg intramuscularly, 10 to 20 μg/ml; and for 400 mg intramuscularly, 2.5 to 10 μg/ml. At 6 hr after single doses, the levels ranged from 1.25 to 10 μg/ml. With multiple doses there was indication of a cumulative effect. A patient with gonococcal arthritis had a synovial fluid level of 2.5 μg/ml 4.5 hr after a dose of 800 mg intramuscularly on the third day of treatment, with a serum level of 10 μg/ml at 1 hr and 2.5 μg/ml at 6 hr.

TABLE 2. *Serum concentrations obtained after various doses of actinospectacin-M141*

| Dose | Amount | Route | Serum antibiotic level | |
|---|---|---|---|---|
| | | | 1 Hr | 6 Hr |
| | | | *μg/ml* | *μg/ml* |
| Single (4 subjects) | 1.0 g | iv | 20-40 | 2.5-10 |
| Multiple (4 patients) | 1.0 g | iv | 20-80 (160)* | 10-40 (80)* |
| Single (4 subjects) | 1.0 g | im | 10-40 | 10 |
| Multiple (2 patients) | 1.0 g | im | 20-80 | 10-20 |
| Single (4 subjects) | 800 mg | im | 10-20 | 2.5-10 |
| Multiple (7 patients) | 800 mg | im | 6-80 | 2.5-40 |
| Single (4 subjects) | 400 mg | im | 2.5-10 | 1.25-2.5 |
| Multiple (22 patients) | 400 mg | im | 5-20 (40)* | 2.5-10 (20)* |

*Highest value represents single patient with azotemia.

Results of the crossover study of actinospectacin-M141 and chloramphenicol are given in Table 3. The serum inhibitory and bactericidal activity of an 800-mg dose of actinospectacin-M141 was about equivalent to 1.0 g of chloramphenicol. Activity equal to 1.0 g of chloramphenicol at 1 hr, but less activity than chloramphenicol at 6 hr, was shown by 400 mg of actinospectacin-M141.

Table 4 shows the results obtained in the treatment of the 35 patients grouped according to diagnosis and causative agent. The

TABLE 3. *Crossover studies comparing serum antibacterial activity of actinospectacin-M141 and chloramphenicol*

| Patient | Antibiotic (given im) | Dose | Serum antibacterial activity* | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | | 1 Hr after dose | | | 6 Hr after dose | | |
| | | | 24-Hr inhibition | 48-Hr inhibition | Bacteri-cidal activity | 24-Hr inhibition | 48-Hr inhibition | Bacteri-cidal activity |
| | | g | | | | | | |
| V.S. | Chloramphenicol | 1.0 | 1:2 | 1:2 | <1:2 | 1:2 | 1:2 | <1:2 |
| | Actinospectacin-M141 | 0.8 | 1:2 | 1:2 | 1:2 | 1:2 | 1:2 | <1:2 |
| J.P. | Chloramphenicol | 1.0 | 1:4 | 1:2 | 1:2 | – | – | – |
| | Actinospectacin-M141 | 0.8 | 1:4 | 1:4 | 1:4 | 1:2 | <1:2 | <1:2 |
| L.L. | Chloramphenicol | 1.0 | 1:2 | <1:2 | <1:2 | 1:2 | <1:2 | <1:2 |
| | Actinospectacin-M141 | 0.4 | 1:2 | 1:2 | <1:2 | <1:2 | <1:2 | <1:2 |

*Against *Paracolobactrum* sp. isolated from blood culture (case 1). In vitro sensitivity, MIC: chloramphenicol, 4 μg/ml; actinospectacin-M141, 3 μg/ml.

result in 26 patients was good or excellent, in eight doubtful, and there was one death attributable to the infection for which the patient was treated.

TABLE 4. *Results of treatment with actinospectacin-M141 in 35 patients*

| Diagnosis | Microorganism | No. of cases | Result | | |
|---|---|---|---|---|---|
| | | | Good | Doubtful | Failure |
| Septicemia | Gram-negative bacilli | 4 | 3 | | 1 |
| Pyelonephritis | Gram-negative bacilli | 10 | 7 | 3 | |
| Pyelonephritis | Gram-positive cocci | 2 | | 2 | |
| Pneumonia | Pneumococcus* | 16 | 13 | 3 | |
| Post-partum endometritis | *Proteus* sp. | 1 | 1 | | |
| Pyogenic arthritis | *Gonococcus*† | 2 | 2 | | |
| Total | | 35 | 26 | 8 | 1 |

*Includes one patient with aspiration (*Proteus* sp.) pneumonia and one with pneumonia of unknown etiology (presumed pneumococcal).

†One patient with positive culture, one presumed clinically with negative cultures.

*Sepsis.* The results in four instances of sepsis are given in Table 5. One patient had two episodes of *Paracolobactrum* sp. septicemia, separated by an asymptomatic period of 4 months, and is presented as cases 1 and 2. During the first admission, when blood cultures were repeatedly positive despite large doses of chloramphenicol, he was given actinospectacin-M141 intramuscularly for 12 days (totaling 28.8 g). On the second admission he was given 30 g intravenously over a 10-day period and 16 g intramuscularly for an additional 10 days. His response was excellent to each course of therapy. The *Paracolobactrum* sp. showed the same in vitro sensitivity on each admission (3.0 μg/ml).

Case 3, a 35-year-old female with *Paracolobactrum* sp. septicemia, made a satisfactory recovery after receiving 1.0 g intravenously every 6 hr for 4 days. This patient had received adequate doses of chloramphenicol and streptomycin without response prior to actinospectacin-M141 therapy.

TABLE 5. *Treatment of septicemia due to gram-negative bacilli with actinospectacin-M141*

| Case | Age | Sex | Complications | Microorganism | MIC (in SAB, pH 7.9) | Dosage (given im) | | | | Remarks |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | Amount | Daily doses | Duration | Total | |
| | *yr* | | | | *μg/ml* | *mg* | *no.* | *days* | *g* | |
| 1 | 32 | M | Sickle cell anemia | *Paracolobactrum* | 3.0 | 400 | 6 | 12 | 28.8 | Serum antibacterial activity (inhibition 1:8) |
| 2 | 32 | M | Sickle cell anemia | *Paracolobactrum* | 3.0 | 1.0 g* | 3 | 10 | 30 | Serum antibacterial activity (bactericidal 1:4) |
| | | | | | | 400 | 4 | 10 | 16 | |
| 3 | 35 | F | Cirrhosis, bleeding varices, hepatic coma, pyelonephritis | *Paracolobactrum* | 2.0 | 1.0 g* | 4 | 4† | 16 | |
| 4 | 70 | M | Cirrhosis, congestive heart failure | *Proteus* sp. | <1.0 | 200 | 4 | 6 | | |
| | | | | | | 200 | 6 | 2.5 | | |
| | | | | | | 500 | 6 | 0.5 | 9.3 | Expired |

*Dose given iv.
†In the early study period, dosage was given iv. Later it was given orally for 17 days with continued favorable results.

In these three successfully treated patients, blood cultures became negative within 48 hr or less.

Case no. 4, a 70-year-old male with septicemia due to a *Proteus* sp. resistant to other antibiotics, showed a poor response and expired. Prior treatment with other antibiotics had been unsuccessful. The total dose of actinospectacin-M141 in this patient was only 9.3 g over 9 days, which we now consider inadequate. *Proteus* sp. was cultured from heart blood post-mortem.

No side effects were noted in these four patients.

*Pyelonephritis.* There were 12 patients with pyelonephritis, one with acute infection, and the others with acute exacerbations of chronic infections (Table 4). Seven patients showed a good response as evidenced by symptomatic remission, clearing of pyuria, and bacilluria. Actinospectacin-M141 dosage ranged from 0.4 to 1.0 g every 6 hr for 6 to 25 days. Of these seven patients, five received 800 mg or more with only two receiving the lower dose.

Response in five patients was considered doubtful as evidenced by symptomatic remission and clearing of pyuria but persistence of bacilluria. Actinospectacin-M141 (400 mg) was given every 6 hr for 9 to 32 days in this group and included the two due to gram-positive microorganisms. Follow-up beyond 10 days could not be obtained in these 12 patients. No side effects were observed.

*Pneumonia.* Fifteen patients with pneumococcal pneumonia and one from whom *Proteus* sp. was isolated were treated with actinospectacin-M141. In this group, 13 had a prompt response, including four with bilateral involvement, and the result was doubtful in three. Actinospectacin-M141 (400 mg) was given every 6 hr for 4 to 9 days to all but one patient who received 800 mg every 6 hr for 7 days.

Of the three in the doubtful group, one with bacteremia had a response in 24 hr but is classified as doubtful because therapy was

inadvertently changed to penicillin after only 2 days. The other two doubtful cases include one with pneumococcal pneumonia superimposed on bronchiectasis and the patient with *Proteus* sp. aspiration pneumonia. No side effects were noted.

*Endometritis.* A patient with postpartum endometritis due to a *Proteus* sp. failed to respond to other antibiotics and was given actinospectacin-M141, 1.0 g intravenously every 8 hr for 2 days followed by 800 mg intramuscularly every 6 hr for 2 days. At this time the patient left the hospital against advice but her response appeared to be excellent. No side effects were observed.

*Pyogenic arthritis.* Two females with gonococcal arthritis received actinospectacin-M141 intramuscularly. One was given 400 mg every 6 hr for 8 days, the other 800 mg every 6 hr for 12 days. Both had a good response. No side effects were seen.

*Side effects.* No local reactions to intramuscular or intravenous actinospectacin-M141 were observed. In this small series no renal, hemopoietic, or hepatic changes were noted. Two patients, still under treatment and therefore not included in this study, developed mild glossitis and stomatitis after 2 weeks of treatment. One of these patients also developed a generalized nonpruritic vesiculo-papular skin reaction. Dosage was 1.0 g every 4 hr intramuscularly in one case and 1.0 g every 6 hr intravenously in the other. In both, the reaction subsided when the agent was discontinued. The patient without dermatitis has since received 400 mg every 6 hr intramuscularly for more than a week with no reaction.

## Discussion

Actinospectacin-M141, administered to 35 patients, yielded good results in 3 of 4 patients with gram-negative septicemia, 7 of 12 with pyelonephritis, 13 of 16 with pneumonia, 1 with endometritis, and 2 with gonococcal arthritis. In vitro sensitivity tests in streptomycin assay broth at pH 7.9 correlated well with the clinical response. Actinospectacin-M141, 0.4 to 1.0 g intramuscularly or intravenously at 4- to 6-hr intervals, is recommended. The side effects included stomatitis and glossitis in two patients, one of whom also had a vesiculo-papular rash. These results with actinospectacin-M141 are promising and further studies are indicated.

## Acknowledgment

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## Literature Cited

Lewis, C., and H. W. Clapp. 1961. Actinospectacin, a new antibiotic. III. In vitro and in vivo evaluation. Antibiotics & Chemotherapy 11:127-133.

Mason, D. J., A. Dietz, and R. M. Smith. 1961. Actinospectacin, a new antibiotic. I. Discovery and biological properties. Antibiotics & Chemotherapy 11:118-122.

Oliver, T. J., A. Goldstein, R. R. Bower, J. C. Holper, and R. H. Otto. 1962. M-141, a new antibiotic. I. Antimicrobial properties, identity with actinospectacin, and production by *Streptomyces flavopersicus*, sp. n. Antimicrobial Agents and Chemotherapy - 1961, p. 495-502.

Sinclair, A. C., and A. F. Winfield. 1962. M-141, a new antibiotic. II. Isolation and characterization and identity with actinospectacin. Antimicrobial Agents and Chemotherapy - 1961, p. 503-506.

# DEGRADATION REACTIONS OF PENICILLINS

## I. Formation Kinetics of Penillic and Penicilloic Acids in Acidic Solutions

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In acid solution penicillins degrade to biologically inactive products at a rate dependent upon the nature of the side chain. At present, penicillins with nonpolar side chains such as *n*-hexyl or benzyl penicillins are representative of the acid-unstable penicillins, whereas the polar α-amino-benzyl penicillin and phenoxymethyl penicillin are representative of the acid-stable penicillins. This is generally interpreted to mean that certain polar side chain groups stabilize the β-lactam ring, thus imparting the acid stability.

One would anticipate, therefore, that the formation of penicilloic acid directly measures lactam lability. However, in acid solutions penillic acid is also formed (Fig. 1).

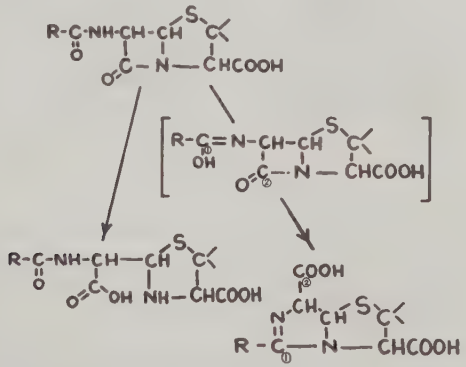


FIG. 1. *Simultaneous formation of penillic acid and penicilloic acid from penicillin.*

The concurrent formation of penillic acid and penicilloic acid occurs in acid solution with the maximum yield of penillic acid in the pH range 2.0 to 3.0 (Florey et al., 1949).

The purpose of the present experiments was to compare the formation rates of the penicilloic and penillic acids of various penicillins, to resolve the role of side chain structure on acid stability.

## Materials and Methods

Arsenomolybdic acid-$HgCl_2$ reagent, as described by Pan (1954) and Nelson (1944), is specific for penicilloic acid. The reagent is reduced to "arsenomolybdate blue" and the absorption is followed at 800 m$\mu$. Penillic acid absorbs maximally at 235 to 237 m$\mu$ and gives no color development with the arsenomolybdate reagent.

Studies with penicillin G and *n*-hexylpenicillin indicated that penillic acid and penicilloic acid were the only significant degradation products in the pH range 1.0 to 4.0.

All penicillins were degraded at 0.001 M, 35 C, and $\frac{\Gamma}{2} = 0.15$. Since the ultraviolet absorption of an expected penillic acid would necessarily be obscured by the absorption of some side chain structures, a general method was adopted as follows: Duplicate samples of the reaction mixture were taken at periodic intervals. To one sample was added the arsenomolybdate-$HgCl_2$ color reagent to give a direct measure of penicilloic acid. The other sample was treated with penicillinase to completion, giving a measure of penicilloic acid plus that formed from remaining penicillin. Penillic acid was then deduced by difference between penicilloic acid plus penicillin, and initial penicillin.

In isolated cases, penillic acid could be determined directly spectrophotometrically. This is aided by the preliminary elimination of penicillin end absorption by use of penicillinase. Penillic acid ultraviolet absorption was measured on a Carey Model 14 spectrophotometer. Colorimetric measurements with the color reagent at 800 m$\mu$ were performed on a Beckman DU spectrophotometer.

## Results

Typical kinetic curves for the formation of penillic acid and penicilloic acid are shown in Fig. 2. Figure 3 illustrates the total formation of the two products as a function of pH.

Since penicillin degrades according to:

$$K = \frac{2.3 \text{ Log } [\text{penicillin}]}{t\ ([\text{penicillin}] - x)} \qquad (1)$$

and the two products are formed first order simultaneously, then:

$$K = k_1 \text{ (penillic acid)} + k_2 \text{ (penicilloic acid)} \qquad (2)$$

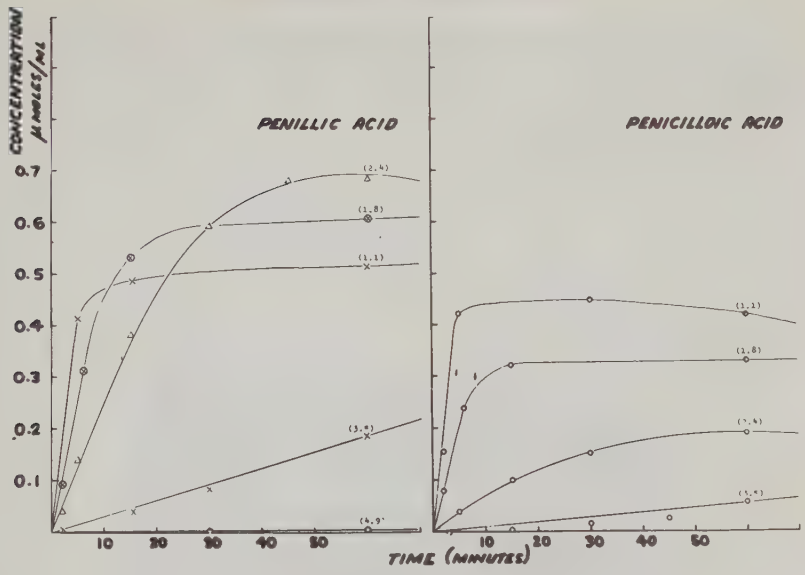



FIG. 2. *Rate of formation of penillic acid and penicilloic acid from benzylpenicillin at various pH ranges.* ($\frac{\Gamma}{2}$ = 0.15, *temperature, 35 C).*

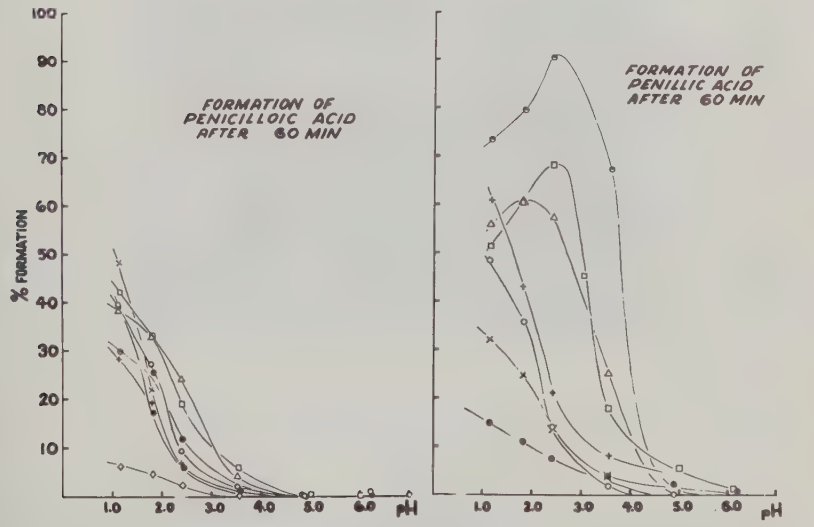



FIG. 3. *Percentage formation of penillic acid and penicilloic acid after 60 min. Symbols:* ● = *n-hexylpenicillin;* Δ = *2,6-dimethoxyphenyl penicillin;* ◐ = *phenoxymethyl penicillin,* □ = *benzylpenicillin, X = o-iodophenyl penicillin;* + = *phenylmercaptomethyl penicillin;* O = *phenoxyisopropyl penicillin;* ◇ = *6-aminopenicillanic acid.*

it follows that x = [penillic acid] + [penicilloic acid] in equation (1). Since the ratio of the two products is constant at any time, $k_1$ and $k_2$ can be evaluated.

$$k_1 = \frac{mK}{m + 1}$$

$$m = \text{constant} = \frac{[\text{penillic acid}]}{[\text{penicilloic acid}]}$$

$$k_2 = \frac{K}{m + 1}$$

A rate comparison between both reactions for six penicillins is shown in Fig. 4.

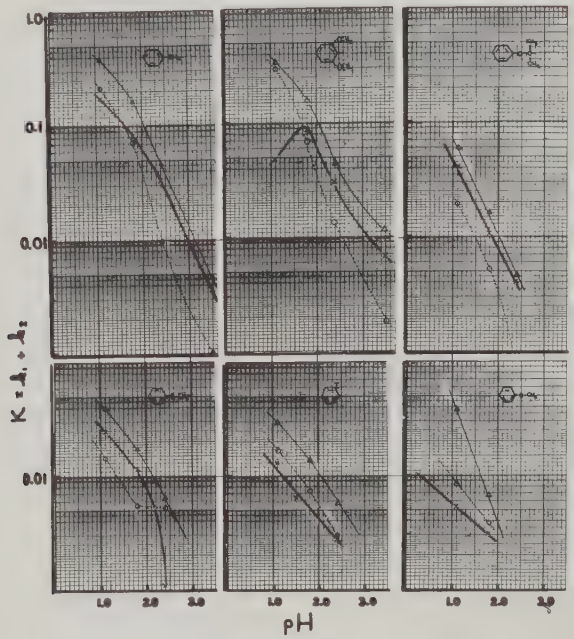



FIG. 4. *Rate constants versus pH for six penicillins. Symbols: Δ = penicillin; O = penicilloic acid; X = penillic acid.*

## Discussion

It was noted that the rate of formation of both penicilloic acid G and penillic acid G increases as pH is lowered (Fig. 2). In the pH

range 1.0 to 2.5, the total amount of penillic acid formed decreases as the pH is lowered. This is because the rate of penicilloic acid formation increases more rapidly as the pH is lowered than does the rate of penillic acid formation, and, consequently, when the penicillin has been completely converted, the amounts of products reflect these relative rates. Figure 3 shows percentage formation, in 60 min, of the two degradation products for seven penicillins and 6-aminopenicillanic acid. It can be seen that penillic acid is formed at a higher pH than is penicilloic acid in all but the most acid-stable penicillins.

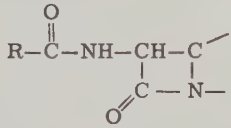
A comparison of several penicillins illustrates the high dependence of penillic acid-formation rate on side chain structure (Fig. 3). The penicilloic acid-formation rate is not so dependent on side chain structure. 6-Aminopenicillanic acid obviously cannot form penillic acid. It is more acid-stable than any penicillin.

The dropoff, at pH 2.5 and below, of the penillic acid curves for acid-unstable penicillins reflects a more rapid rate of penicilloic acid formation at lower pH values (Fig. 3). It does not imply a lower rate of penillic acid formation at lower pH values.

A comparison of the rates of formation of the two penicillin degradation products with the rate of penicillin loss is shown in Fig. 4. The closer the k versus pH curve for a product approaches the K versus pH curve for penicillin loss, the more dependent is the penicillin loss on that particular product formation, at a given pH.

It can be seen that benzylpenicillin, 2,6-dimethoxyphenyl penicillin, and phenoxyisopropyl penicillin are acid-unstable. This instability is due primarily to the high rate of formation of penillic acid. This is seen by making comparisons at pH 2.0. Phenoxymethyl penicillin, on the other hand, forms penillic acid at a much slower rate, thus imparting acid stability. Phenylmercaptomethyl penicillin and *o*-iodophenylpenicillin are intermediate in acid stability and in the rate of formation of penillic acid.

The hydrolysis of penicillin to penicilloic acid must involve the reaction penicillin + HOH $\longrightarrow$ penicilloic acid and the reaction must involve direct attack of the $\beta$-lactam bond by HOH. The lability of the bond is of course somewhat influenced by polarization through such atoms as intervene between the side chain R and the C-N bond.



It has been difficult to conceive how the remarkable effects of side chain structure on stability of penicillins could be produced in this manner by stabilizing the $\beta$-lactam C-N bond.

A very different situation arises in the conversion of penicillins to penillic acid.

Here, the reaction presumably involves a direct intramolecular rearrangement in which a $C_1$-OH bond reacts with a $C_2$-N bond to yield $C_1$-N and $C_2$-OH. This can be seen by reference to Fig. 1, in which the conversion can best be pictured as proceeding from the electromeric form in brackets. An atomic model of such a structure shows that the mutual relations of the bonds and atoms involved makes this appear feasible.

The occurrence of such a structure and the rate of any reaction directly involving the carbonyl group on which this side chain is attached should be very directly affected by side chain polarization. Furthermore, the effect of side-chain polarization on the contribution of the bracketed electromer is such as to produce a reduction in this form by just those influences which lead to acid stability of penicillins.

No detailed examination of k vs. pH has been made; accordingly, the expected influence of pK of the products on these reaction rates could not be examined.

## Literature Cited

Florey, H. W., E. Chain, N. G. Heatley, M. A. Jennings, A. G. Sanders, E. P. Abraham, and M. E. Florey. 1949. Antibiotics, vol. 2, p. 890. Oxford University Press, London.

Nelson, N. 1944. A photometric adaption of the Somogy method for the determination of glucose. J. Biol. Chem. **153**:375-380.

Pan, S. C. 1954. Simultaneous determination of penicillin and penicilloic acid in fermentation samples by a colorimetric method. Anal. Chem. **26**:1438-1444.

# PREPARATION AND CHARACTERIZATION OF THE *CIS* AND *TRANS* FORMS OF 6-(STYRYLMERCAPTOACETAMIDO) PENICILLANIC ACID

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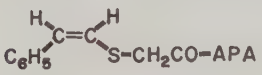
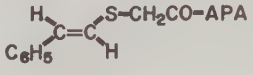
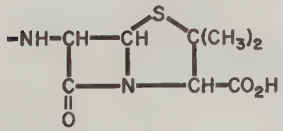
The purpose of this paper is to report chemical and biological data which have been accumulated on *cis* and *trans* forms of 6-(styrylmercaptoacetamido)penicillanic acid (SM) and to discuss structure-activity relationships in these novel penicillins (Fig. 1). Chemically, the subject compounds represent unique geometric isomers of a specific vinylog of 6-(phenylmercaptoacetamido)penicillanic acid



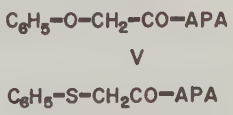

cis-SM



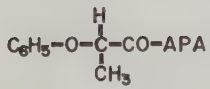

trans-SM



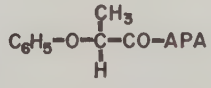

APA




PM




D-PE




L-PE

FIG. 1. *Structures of various penicillins. Abbreviations: SM = 6-(styrylmercaptoacetamido) penicillanic acid; APA = 6-aminopenicillanic acid moiety; V = 6-(phenoxyacetamido) penicillanic acid; PM = 6-(phenylmercaptoacetamido) penicillanic acid; PE = 6-(α-phenoxypropionamido) penicillanic acid (phenethicillin).*

(PM). This penicillin is a simple sulfur-analogue of the well-known 6-(phenoxyacetamido)penicillanic acid (V). Other structural departures from penicillin-V have included variants containing optically isomeric "side-chains," such as the D- and L-phenethicillins (PE), which are pertinent to comparisons of different types of stereoisomeric effects. Two publications appeared recently which reported extensive in vitro comparisons of various penicillins including PM, V, D-PE, and L-PE (Wallmark and Finland, 1961; McCarthy, Wallmark, and Finland, 1961).

## Materials and Methods

*Preparation and characterization of cis and trans isomers of styrylmercaptoacetic acid.* Holmberg's (1950) synthesis of "A" and "B" forms of the subject acid, also termed styrylthioglycolic acid, was successfully repeated. The preparative procedure involved reaction of phenylacetylene and thioglycolic acid (Fig. 2a) and separation of the resulting acidic products by means of differential solubilities of their barium salts. After purification, the free isomeric acids were crystallized from benzene-hexane mixtures. The relatively more soluble acid, "A," and its companion, "B," exhibited remarkably similar melting points within the range of 88 to 90 C. Various admixtures, however, showed marked melting point depressions (1:1 mixture, mp 64 to 67 C). Our melting point data as well as elementary analyses and titration data, which agreed with Holmberg's results, clearly showed that two $C_{10}H_{10}O_2S$ acids were obtained.

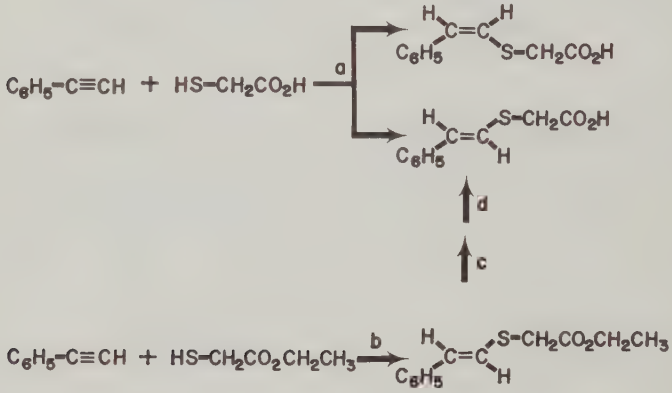



FIG. 2. *Syntheses of cis and trans-styrylmercaptoacetic acids.*

Spectroscopic measurements were initiated on "A" and "B" to further characterize these isomeric acids. The "fingerprint" region

in the infrared spectrum of each acid (Fig. 3) was distinctly different; intense absorptions at 12.8 $\mu$ for "A" and 10.7 $\mu$ for "B" were especially useful for diagnostic purposes. The ultraviolet-absorption spectra and intensities exhibited by "A" and "B" were significantly different (Fig. 4). The hypsochromic qualitative and hypochromic quantitative ultraviolet-absorption properties of "A" ($\lambda$ max 282 m$\mu$; $\epsilon$ 16,500) relative to those of "B" ($\lambda$ max 287 m$\mu$; $\epsilon$ 19,400) provided evidence to support a *cis* configuration assignment fo "A" and, conversely, a *trans* assignment to "B".

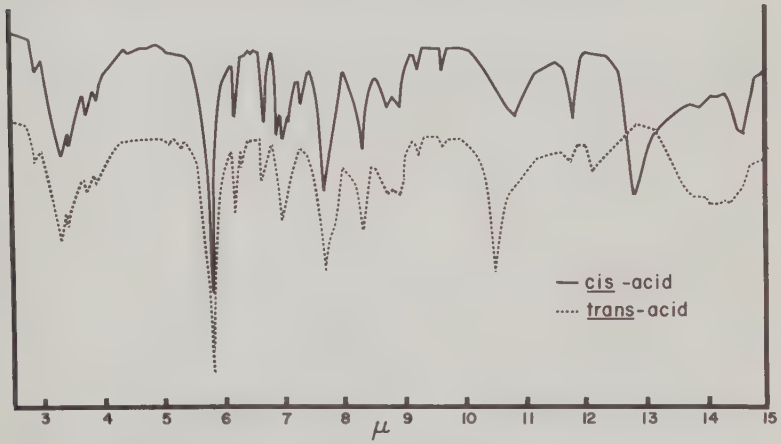



FIG. 3. *Infrared spectra in methylene chloride solutions of isomeric styrylmercaptoacetic acids.*

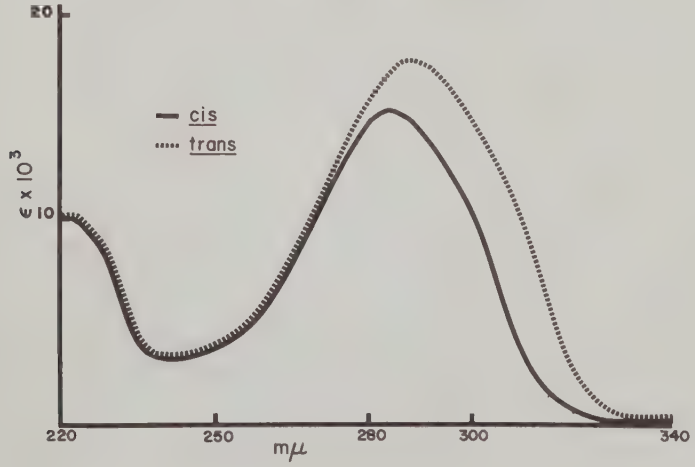



FIG. 4. *Ultraviolet absorption spectra of isomeric styrylmercaptoacetic acids (alcohol solutions).*

*Alternative preparative method.* The use of ethyl thioglycolate in place of thioglycolic acid for reaction with phenylacetylene favored the formation of a *trans* product. The resulting ester was recovered by distillation in vacuo, saponified with methanolic sodium hydroxide, and the crystalline sodium salt was then converted to pure *trans*-acid (cf. Fig. 2b to d). Although extra steps were involved, this method was preferred to Holmberg's procedure for the preparation of relatively large quantities of *trans*-styrylmercaptoacetic acid.

1) Preparation of ethyl *trans*-styrylmercaptoacetate: Ethyl thioglycolate (100.0 g) was cautiously added to phenylacetylene (106.4 g) in a 500-ml round-bottom flask equipped with a reflux condenser. A vigorous exothermic reaction followed and refluxing became evident several minutes after the completion of the mixture. The reaction was allowed to proceed without any external heating or cooling and after 30 min refluxing subsided. The reaction was allowed to stand overnight during which time the mixture cooled to room temperature. The reaction flask was then equipped with a short-path distillation head and heated in an oil bath under high vacuum. The main distillate (bp 124 to 125 C at 0.2 to 0.25 mm) condensed as a pale yellow, free-flowing liquid (141 g).

2) Preparation of *trans*-styrylmercaptoacetic acid: A stirred solution of ethyl *trans*-styrylmercaptoacetate (119 g) in methanol (715 ml) was treated dropwise over a period of 0.5 hr with 360 ml of a 13% sodium hydroxide in water-methanol (1:2) solution. Sodium *trans*-styrylmercaptoacetate gradually crystallized from the saponification reaction mixture and after 1 hr the suspension was filtered. The crystals were washed with methanol (120 ml) and dried (80.5 g). The product was dissolved in water (1,770 ml) and the stirred solution was acidified gradually over a period of 0.5 hr with 130 ml of 25% aqueous acetic acid. Crystals appeared and were recovered after 1 hr (43.8 g). A second crop (6.7 g) was obtained following further acidification. Both products recrystallized from hot hexane afforded needles (mp 83 to 89 C), which were characterized as pure *trans*-styrylmercaptoacetic acid.

*Preparation of cis and trans forms of 6-(styrylmercaptoacetamido)penicillanic acid.* A solution of *trans*-styrylmercaptoacetic acid (80 g) in dry benzene (400 ml) was treated with excess oxalyl chloride (100 g) at room temperature, exercising the prescribed precautions for handling this hazardous reagent. The reaction (Fig. 5a) proceeded overnight, after which completion was evidenced by absence of gas evolution. The solvent and excess oxalyl chloride were removed in vacuo. The residual *trans*-styrylmercaptoacetyl chloride was then added over a 1 hr period to a stirred solution of 104 g of 6-aminopenicillanic acid (Fig. 5b) dissolved in 2,700 ml of water containing 173 g of potassium bicarbonate at 8.5 C. After 2 hr total reaction time, the temperature had risen to 21 C. The reaction products were then fractionated by a potassium acetate salting-out

technique. After the addition of 860 g of potassium acetate, most of the potassium *trans*-styrylmercaptoacetate by-product (20.3 g) was precipitated. After its removal, an additional 500 g of potassium acetate was used to induce crystallization of the desired penicillin potassium salt (91 g). This material was satisfactorily recrystallized from water-methylethylketone.

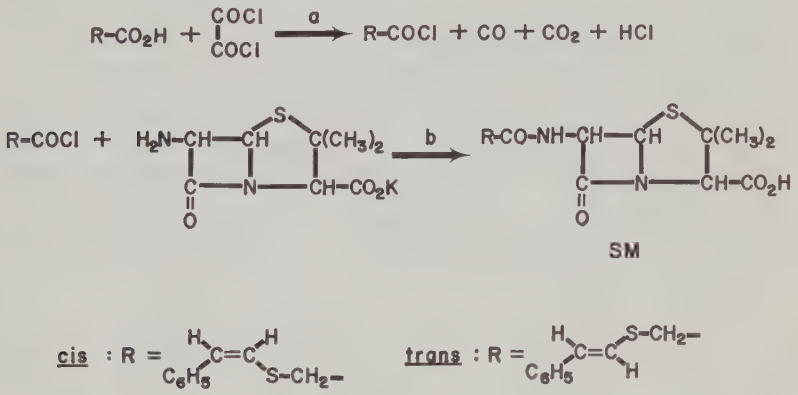

R-CO2H + COCl COCl a R-COCl + CO + CO2 + HCl
R-COCl + H2N-CH-CH C(CH3)2 b R-CO-NH-CH-CH C(CH3)2
C-N-CH-CO2K
O
C-N-CH-CO2H
O
SM
cis : R = H C=C H C6H5 S-CH2-
trans : R = H C=C S-CH2- C6H5 H

FIG. 5. *Preparation of isomeric forms of 6-(styrylmercaptoacetamido) penicillanic acid.*

The above procedure starting with *cis*-styrylmercaptoacetic acid in place of the *trans*-acid gave the crystalline potassium salt of *cis*-6-(styrylmercaptoacetamido)penicillanic acid.

*Biological studies.* The various microorganisms used are routinely carried in our stock culture collection. Standard laboratory methods were employed which have been previously described in detail (English and McBride, 1959).

Human blood serum level investigations were conducted with the various penicillin potassium salts in hand-filled hard gelatin capsules, each containing 250 mg of free penicillin activity.

Fasting healthy adult males were used as test subjects. Blood samples of 10 to 15 ml each were obtained from forearm veins and collected in sterile centrifuge tubes. Samples were refrigerated immediately and delivered to the assay laboratory where the tubes were centrifuged and the serum was recovered by decantation. Whenever possible, the sera were assayed immediately; otherwise they were frozen and assayed the next day. The standard penicillin cylinder plate method, employing *Sarcina lutea* (Grove and Randall, 1955), was used; each penicillin under test was assayed against its own standard. Common and modified (English et al., 1953) twofold serum-dilution assays were also conducted utilizing *Staphylococcus aureus* and *Streptococcus pyogenes*.

## Results

*Chemical and physical characterization of the crystalline potassium salts of cis and trans forms of SM.* Elemental analyses were consistent with $C_{18}H_{19}N_2S_2O_4K$ compositions for the subject compounds as were chemical penicillin assays which calculated 1,380 international units per milligram based on stoichiometric projection from sodium penicillin G. The crystalline potassium salts of the SM isomers exhibited satisfactory stability in the dry form and were freely soluble in water; stabilities in acid solution were comparable to penicillin PM, that is, considerably more stable than penicillin G but somewhat less so than penicillin V. Optical rotations in water were similar: potassium *cis*-SM, $[\alpha]_D$ + 220 ± 10, potassium *trans*-SM, $[\alpha]_D$ + 210 ± 10. Infrared curves (Fig. 6) illustrated $\beta$-lactam absorption near 5.6 $\mu$, characteristic of all penicillins, and other bands served to clearly differentiate the *cis* and *trans* isomers of SM. Characteristic ultraviolet absorption of the "precursor" acids carried over to their respective penicillins.

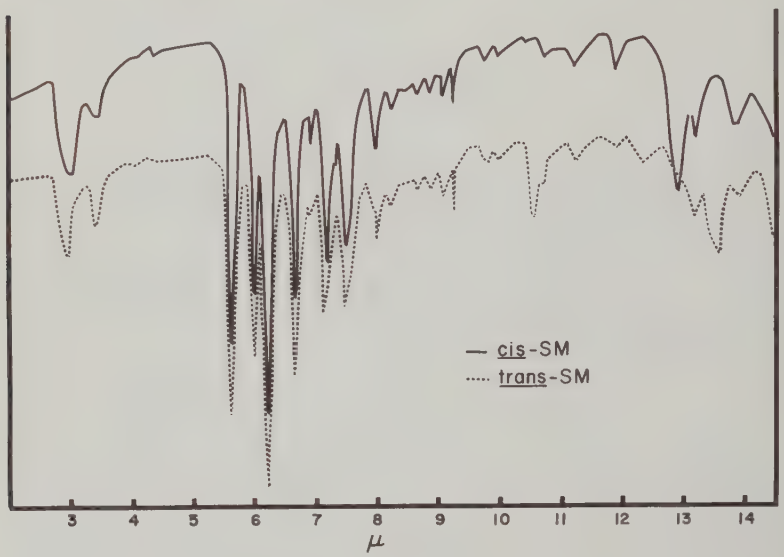



FIG. 6. *Infrared spectra of the potassium salts of isomeric 6-(styrylmercaptoacetamido) penicillanic acids in potassium bromide pellets.*

*Biological studies.* The in vitro antimicrobial activities of *cis*-SM and *trans*-SM are presented in Table 1.

Acute intravenous toxicities observed in mice were similar and comparable to those of the well-known penicillins: *cis*-SM, $LD_{50}$ = 300 mg/kg; and *trans*-SM, $LD_{50}$ = 320 mg/kg.

TABLE 1. *In vitro antimicrobial activities of 6-(styrylmercaptoacetamido) penicillanic acid*

| Microorganism | Minimal inhibitory concentration | |
|---|---|---|
| | *Cis*-SM | *Trans*-SM |
| | *μg/ml* | *μg/ml* |
| *Staphylococcus aureus* | 0.0156 | 0.0156 |
| *Staphylococcus aureus* 376* | >100 | >100 |
| *Staphylococcus aureus* 400* | >100 | >100 |
| *Streptococcus pyogenes* | <0.0019 | 0.0039 |
| *Streptococcus faecalis* | 0.0625 | 0.0025 |
| *Diplococcus pneumoniae* | 0.39 | 0.25 |
| *Erysipelothrix rhusiopathiae* | 0.0312 | 0.0625 |
| *Corynebacterium diphtheriae* | 0.0156 | 0.0312 |
| *Listeria monocytogenes* | 0.0625 | 0.19 |
| *Bacillus subtilis* | 0.0625 | 0.0156 |
| *Streptococcus agalactiae* | 0.0078 | 0.0156 |
| *Brevibacterium ammoniagenes* | 0.25 | 0.0625 |
| *Aerobacter aerogenes* | >100 | >100 |
| *Escherichia coli* | >100 | >100 |
| *Proteus vulgaris* | >100 | >100 |
| *Pseudomonas aeruginosa* | >100 | >100 |
| *Salmonella typhosa* | >100 | 50 |
| *Salmonella gallinarum* | >100 | 25 |
| *Klebsiella pneumoniae* | 50 | 50 |
| *Neisseria gonorrhoeae* | 0.0625 | 0.0625 |
| *Bordetella pertussis* | 0.78 | 3.12 |
| *Shigella sonnei* | 100 | >100 |
| *Bordetella bronchiseptica* | 100 | >100 |
| *Actinobacillus mallei* | 12.5 | 50 |
| *Vibrio comma* | 1.56 | 0.78 |
| *Pasteurella multocida* | 0.0312 | 0.0312 |
| *Candida albicans* | >100 | >100 |
| *Streptococcus pyogenes* 98 | 0.0156 | 0.0625 |
| *Staphylococcus aureus* 209 | <0.0019 | <0.0019 |
| *Streptococcus pyogenes* 98† | 0.0156 | 0.39 |
| *Staphylococcus aureus* 209† | 0.25 | 0.39 |
| *Staphylococcus aureus*† | 0.25 | 0.39 |
| *Streptococcus pyogenes*† | 0.0156 | 0.0156 |
| *Mycobacterium* 607 | >100 | >100 |

*Resistant to high concentrations of penicillin G.
†With serum.

Activities against experimental *S. aureus* infections in mice in terms of $ED_{50}$ (mg/kg) were: *cis*-SM, 25 (orally) and 13 (subcutaneously), and *trans*-SM, 15 (orally) and 7.5 (subcutaneously); against *S. pyogenes* the activity of *trans*-SM improved to 2.5 (orally) and 1.4 (subcutaneously).

Oral absorption studies in dogs revealed comparable serum antibiotic levels for *cis*-SM and *trans*-SM which were lower than those observed for penicillin V (Fig. 7). Studies in humans revealed an entirely different picture, in which *trans*-SM promoted significantly higher antibiotic serum levels than *cis*-SM and penicillin V (Fig. 8).

Direct comparison of *trans*-SM with penicillin PM showed greater weight absorption at all time periods for *trans*-SM. Relative results expressed in terms of serum activity dilutions varied depending on the organism and assay technique used (Table 2).

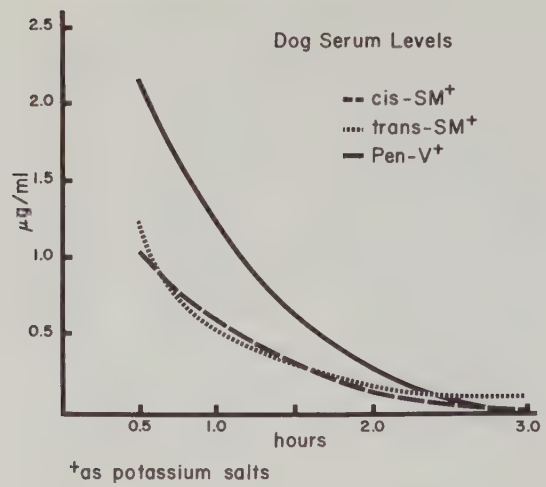



FIG. 7. *Average serum level patterns after 10-mg/kg oral doses of various penicillins (five dogs per test).*

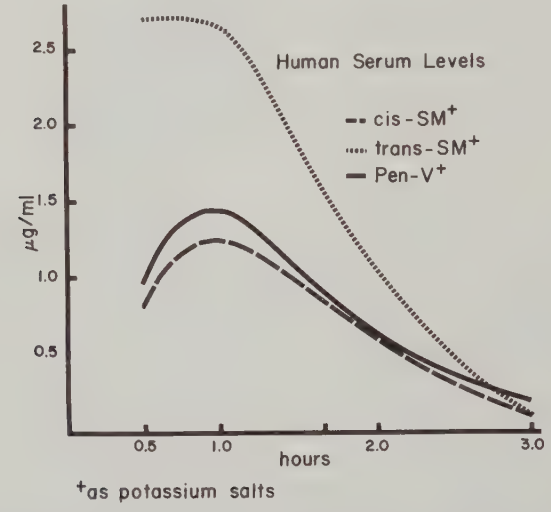



FIG. 8. *Average serum level patterns after 150-mg oral doses of various penicillins in 24 normal adults.*

TABLE 2. *Human serum level comparisons with 250 mg of antibiotic administered orally*

| Antibiotic | Assay organism | Method† | *Average weight levels (μg/ml)* 0.5 hr | 1 hr | 2 hr | 4 hr | 6 hr |
|---|---|---|---|---|---|---|---|
| Penicillin SM | | | 3.155 (15/15)* | 3.597 (15/15) | 1.578 (15/15) | 0.214 (15/15) | – (3/15) |
| Penicillin PM | | | 1.881 (15/15) | 2.186 (15/15) | 1.248 (15/15) | 0.105) (15/15) | (3/15) |
| | | | | *Serum dilutions* | | | |
| Penicillin SM | *S. aureus* | Common | 1:33 | 1:40 | 1:11 | 0 | 0 |
| | | English | 1:7.6 | 1:7.6 | 1:4.1 | 0 | 0 |
| | *S. pyogenes* | Common | 1:ca.1900 | 1:ca.1900 | 1:870 | 1:41 | 1:4 |
| | | English | 1:738 | 1:370 | 1:313 | 1:28 | 1:2 |
| Penicillin PM | *S. aureus* | Common | 1:42 | 1:34 | 1:19 | 1:1 | 0 |
| | | English | 1:21 | 1:24 | 1:14 | 1:1 | 0 |
| | *S. pyogenes* | Common | 1:ca.747 | 1:ca.751 | 1:359 | 1:7 | 1:0.4 |
| | | English | 1:641 | 1:465 | 1:211 | 1:6 | 1:1 |

*The ratio in parentheses is the number producing measurable serum activity in the 15 volunteers.

†Common: brain heart infusion assay medium; English: brain heart infusion + 25% serum assay medium.

## Discussion

Rigorous identification criteria for the isomeric forms of styrylmercaptoacetic acid and their derivatives were accomplished by spectroscopic means. Grummitt and Christoph (1951) have described the isomers of 1-phenyl-1,3-butadiene (*cis:* λ max 267 mμ and *trans:* λ max 280 mμ) and have put forth ample arguments for their stereoconfigurational assignments, which included chromophore length and coplanarity considerations. Spectroscopic principles applicable to $C_6H_5$-CH=CH-CH=$CH_2$ similarly affect the isosteric chromophore, $C_6H_5$-CH=CH-S-, present in the pertinent compounds described in this paper.

In vitro antimicrobial studies revealed that both penicillins studied exhibited excellent potencies within spectra comparable to those described for the penicillins V, PM, and D, L-PE (Wallmark and Finland, 1961; McCarthy et al., 1961; English and McBride, 1961). The *cis* and *trans* forms of SM did not vary markedly in their in vitro activities against various sensitive bacteria. This phenomenon is in contrast to the rather consistently greater in vitro activity reported for L-PE relative to D-PE and denotes less in vitro specificity toward the geometric isomers than toward optical isomers, in these cases.

Absorption studies in dogs and man disclosed notable species differences (Fig. 7 and 8; Table 2). The *trans* form of SM was

measurably superior to *cis*-SM, PM, and V in terms of promoting antibiotic serum levels following ingestion by human subjects. The absorption study results clearly illustrate the necessity for evaluation of pure chemical entities in more than one animal species.

## Acknowledgments

We are indebted to Mark Jefferson, Constantine Zervos, Matthew Calvin, Roy Mackie, and B. Ryan for capable technical assistance. We wish to acknowledge acute toxicity studies performed by C. Delahunt, human serum penicillin assays by R. Kersey, and physical measurements by R. Wagner. The help of R. Drinkard and C. Maxwell in developing procedures for the practical preparation of large quantities of "precursor" acids is especially appreciated. Our thanks are also due to B. Sobin, A. Finlay, and E. M. Weber for their interest.

## Literature Cited

English, A. R., M. F. Field, J. Reilly, J. McNeirney, and S. Y. P'an. 1953. Magnamycin, preliminary studies on absorption and execretion after single dosage. Antibiotics & Chemotherapy 3:397-313.

English, A. R., and T. J. McBride. 1959. Laboratory evaluation of partially-acetylated esters of oleandomycin. Proc. Soc. Exptl. Biol. Med. 100:880-884.

English, A. R., and T. J. McBride. 1962. Bacteriological comparison of L- and D-phenethicillin. Antimicrobial Agents and Chemotherapy - 1961, p. 636-641.

Grove, D. C., and W. A. Randall. 1955. Assay methods of antibiotics a laboratory manual. Medical Encyclopedia, Inc., New York.

Grummitt, O., and F. J. Christoph. 1951. Geometric isomers of 1-phenyl-1,3-butadiene. J. Am. Chem. Soc. 73:3479-3484.

Holmberg, V. B. 1950. Über styrylthioglykolsäuren. Arkiv Kemi 2:567-579.

McCarthy, C. G., G. Wallmark, and M. Finland. 1961. In vitro activity of various penicillins. Am. J. Med. Sci. 241:143-159.

Wallmark, G., and M. Finland. 1961. Comparative activity of various penicillins against penicillinase-producing and non-penicillinase-producing staphylococci. Proc. Soc. Exptl. Biol. & Med. 106:78-85.

# IN VITRO EXAMINATION OF LEVOPROPYLCILLIN

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Since the announcement by Batchelor et al. (1959) of the isolation of 6-aminopenicillanic acid and the preparation of a number of new types of penicillins therefrom, problems long associated with the penicillin antibiotics have suddenly taken on a new glamour and attraction for researchers in laboratories and clinics. So far, however, the "ideal" antibiotic has yet to be discovered.

Levopropylcillin is a new type of penicillin that is one isomeric form of the penicillin announced publicly by Williamson, Morrison, and Stevens (1961). The structural formula of levopropylcillin is presented in Fig. 1. Chemically, this penicillin is 6-(*l*-α-phenoxy-*n*-butyramido) penicillanic acid.

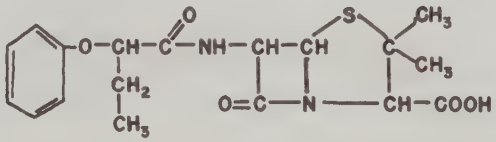


FIG. 1. *Structural formula of levopropylcillin.*

This paper presents data which suggest that penicillinase resistance may not be required for a penicillin antibiotic to inhibit penicillinase-producing resistant staphylococci.

## Materials and Methods

*Resistant staphylococci.* Cultures of penicillin-resistant staphylococci were requested from a number of clinical investigators so that our laboratory tests of antibacterial activity could be based upon resistant strains representative of those being isolated in hospitals all over the country. All cultures were tested for penicillinase production as they were received, and all were found to be penicillinase producers. Penicillinase activity was determined in centrifuged broth

and sonic-treated cells by a chemical assay, which measured the amount of $\beta$-lactam present as a function of time. The assay was that of Ford (1947), which uses hydroxylamine as the reagent.

Minimal inhibitory levels of antibiotics were determined by the gradient plate method fashioned after that described by Bryson and Szybalski (1952). We have found this the most satisfactory method.

The inhibition pattern, or profile, if you will, was obvious from a glance at the plate, and when the procedure was standardized with adequate controls, the test was reproducible from day to day and week to week. The most important advantage of the gradient plate for screening antibiotics was the ease of detecting small differences between different antibiotics that would have been missed by a plus-minus growth-measuring system.

Standard tube dilutions were run in brain heart infusion broth.

The cultures of penicillin-resistant staphylococci were treated uniformly in the following way. (i) Cultures, as received, were inoculated into brain heart infusion broth and were grown at 37 C for 18 to 20 hr. (ii) The broth culture (0.1 ml) was then spread evenly over the surface of a *Staphylococcus* no. 110 (Difco) slant and was incubated at 37 C for 48 hr. (iii) Water (2.0 ml) was then added to the 110 slant, and the growth was thoroughly scraped off the surface and vigorously suspended in water. (iv) A portion (0.1 ml) of this water suspension was then streaked evenly on the surface of a Penassay agar slant and incubated at 37 C for 24 hr. (Penassay agar: Bacto peptone, 6.0 g/liter; N-Z-case, 4.0 g/liter; yeast extract, 6.0 g/liter; beef extract, 1.5 g/liter; and Bacto agar, 20.0 g/liter.) (v) After 24 hr on Penassay agar, 5.0 ml of water were added to the surface and the tube was agitated to suspend the cells. This tube was then refrigerated at 5 C for 5 days. The resistant staphylococcal cells were then usable for streaking the gradient plates on the fifth and sixth days.

## Results

*Antistaphylococcal activity.* When levopropylcillin was assayed biologically by the standard disc, *S. aureus* 209P diffusion assay, it had an activity of 515 units per mg expressed as penicillin G. An agar plate serial-dilution test measured the sensitivity of *S. aureus* 209P as 0.05 $\mu$g/ml. It is interesting to note that the penicillin prepared from the *d*-phenoxybutyric acid, which we will call dextropropylcillin, had the same sensitive staphylococcal (209P) activity as did the levopropylcillin.

The minimal inhibitory concentration for some well-known antibiotics, against a variety of penicillin-resistant staphylococci, was determined using the gradient plate test (Table 1).

TABLE 1. *Minimal inhibitory concentrations against resistant staphylococci**

| Culture no. | Source | Penicillin G | Phenethicillin | Levopropylcillin |
|---|---|---|---|---|
| | | *μg/ml* | *μg/ml* | *μg/ml* |
| V-1 | Dallas | 14 | 2 | 1 |
| V-6 | Dallas | 49 | 14 | 3 |
| V-8 | Dallas | 73 | 24 | 5 |
| V-10 | Dallas | 31 | 5 | 2 |
| V-12 | Washington, D. C. | 36 | 6 | 3 |
| V-18 | Washington, D. C. | 50 | 7 | 2 |
| V-28 | New York City | 60 | 9 | 4 |
| V-30 | New York City | 54 | 11 | 5 |
| V-32 | New York City | 64 | 12 | 6 |
| V-37 | San Francisco | 35 | 11 | 4 |
| V-38 | San Francisco | 36 | 6 | 1 |
| V-39 | San Francisco | 53 | 15 | 7 |
| V-40 | Minneapolis | 21 | 4 | 1 |
| V-41 | Minneapolis | 31 | 9 | 3 |
| V-42 | Minneapolis | 80 | 22 | 7 |
| V-68 | Philadelphia | 57 | 14 | 10 |
| V-69 | Cincinnati | 97 | 27 | 5 |
| V-71 | Cincinnati | 104 | 28 | 8 |
| V-81 | Detroit | 200 | 65 | 27 |
| V-84 | Detroit | 126 | 43 | 32 |
| V-85 | Detroit | 50 | 14 | 4 |
| V-89 | Cincinnati | 114 | 43 | 13 |
| V-90 | Cincinnati | 120 | 43 | 12 |
| V-94 | Cincinnati | 47 | 10 | 2 |
| V-102 | Seattle | 200 | 27 | 8 |
| V-105 | Seattle | 70 | 21 | 10 |
| V-110 | Rochester, Minn. | 200 | 29 | 5 |
| V-113 | Rochester, Minn. | 80 | 21 | 5 |
| V-118 | Salt Lake City | 200 | 200 | 200 |
| V-128 | Miami | 35 | 6 | 2 |
| V-135 | Seattle | 65 | 19 | 11 |
| V-138 | Seattle | 2 | .1 | .2 |
| V-143 | Seattle | 90 | 19 | 9 |
| Kelly | Seattle | 49 | 16 | 7 |
| 60-1 | Boston | 45 | 9 | 2 |
| H-400 | Boston | 38 | 11 | 5 |

*MIC's are for three antibiotics tested by a gradient plate method against penicillin-resistant staphylococci. This method permits a demonstration of the relative degrees of activity of the antibiotics against resistant staphylococci.

Results with a standard serial-dilution tube test differed with respect to actual end points from those of the gradient plates, but the relative positions (or activities) of the antibiotics were the same (Table 2).

*Effect of size of inoculum.* A great deal of controversy is still centered around the size of the inoculum and its effect on the validity of the test results. It is assumed that this "validity" refers to

TABLE 2. *Serial dilution results**

| Culture no. | Penicillin G | Phenethicillin | Levopropylcillin |
|---|---|---|---|
| | *μg/ml* | *μg/ml* | *μg/ml* |
| V-30 | >200 | 200 | 50 |
| V-32 | >200 | 100 | 25 |
| V-41 | >200 | 100 | 25 |
| V-84 | >200 | 50 | 6 |

*Serial dilution brain heart infusion broth tests on four of the strains of resistant staphylococci.

whether or not the results can be translated into terms of clinical effectiveness. The size of the inoculum should logically correspond to conditions that may be encountered in vivo during an infection. The effect of the cultural history of an organism upon a series of experimental results is a well-known experimental variable, yet it is one which is quite often given only minor consideration. Emphasis upon the size of the inoculum seems a little out of place when the test system utilized is completely unrelated to an in vivo environment. More important than any other experimental variable is the inclusion of a control antibiotic which has a known clinical effectiveness. The effect of inoculum variation on the antibiotic sensitivity of the test organisms, as obtained from gradient plate results, is summarized in Table 3.

TABLE 3. *Effect of inoculum size**

| | | Organism | | | |
|---|---|---|---|---|---|
| Antibiotic | Inoculum dilution | V-30 | V-32 | V-41 | V-84 |
| Penicillin G | 1:50 | 73 | 46 | 32 | 59 |
| Penicillin G | 1:25 | 52 | 57 | 33 | 58 |
| Penicillin G | 1:10 | 85 | 88 | 96 | 90 |
| Phenethicillin | 1:50 | 6 | 21 | 7 | 16 |
| Phenethicillin | 1:25 | 4 | 19 | 10 | 17 |
| Phenethicillin | 1:10 | 35 | 58 | 27 | 28 |
| Levopropylcillin | 1:50 | 2 | 5 | 2 | 8 |
| Levopropylcillin | 1:25 | 9 | 12 | 6 | 8 |
| Levopropylcillin | 1:10 | 11 | 18 | 7 | 13 |

*Values given in μg/ml required to inhibit growth on a gradient plate of the resistant staphylococci listed. A water suspension of the staphylococci containing $10^{13}$ organisms per ml was diluted as indicated before streaking the individual gradient plates

*Serum effect.* The effect of 50% normal human serum upon the antibiotic activity of levopropylcillin is shown in Table 4.

The in vitro diminution of antibiosis sometimes encountered in the evaluation of an antibiotic in serum, a material which does not

exist as such in vivo, has generally been termed "serum binding" or "serum inactivation" of the antibiotic in question. It is noted here that the apparent effect of serum on an antibiotic must be considered only in terms of the test organism being used. Any test system, measuring the effect of an antibiotic upon an organism and in the presence of serum, should be termed at least a three-phase system and not a two-phase system as it is usually considered. No "serum binding" has been noted in our laboratories when *Bacillus subtilis*, instead of *S. aureus*, is used as the test organism for penicillin G or penicillin V. It is conceivable that a different mechanism of antibiotic action could take place in the presence of serum, depending on the effect of serum on the test organism being used.

TABLE 4. *Activity of levopropylcillin in human serum**

| Antibiotic | Serum | Organism | | | |
|---|---|---|---|---|---|
| | | V-30 | V-32 | V-41 | V-84 |
| Penicillin G | (−) | 61 | 61 | 40 | 49 |
| Penicillin G | (+) | 84 | 93 | 43 | 56 |
| Phenethicillin | (−) | 6 | 12 | 9 | 13 |
| Phenethicillin | (+) | 33 | 41 | 18 | 39 |
| Levopropylcillin | (−) | 3 | 10 | 8 | 6 |
| Levopropylcillin | (+) | 7 | 27 | 11 | 11 |

*Values are in terms of μg/ml to inhibit the resistant staphylococci as determined on gradient plates.

*Penicillinase stability.* The most interesting aspect of levopropylcillin is in the area of penicillinase stability. The phenoxypropylcillin prepared from either the *d* or the *l* acid is more stable to *B. cereus*-produced penicillinase (Schenley Industries, Inc., New York, N.Y., or Riker Laboratories, Inc., Los Angeles, Calif.) than penicillin G or V, but only the penicillin prepared from the *d* acid demonstrated any increased stability when tested against staphylococcal penicillinase. The penicillin prepared from the *l* acid, levopropylcillin, was, however, more active against penicillinase-producing staphylococci. If the test for antiresistant staphylococcal activity had been designed as a test for penicillinase stability, levopropylcillin would have been missed. Table 5 compares some antiresistant staphylococcal activities of the two penicillins, dextropropylcillin, and levopropylcillin. These data demonstrate that levopropylcillin is the most active.

Figure 2 is a graph of the data obtained when both levopropylcillin and dextropropylcillin were compared with penicillin G as to their stabilities in penicillinase-containing broth derived from a resistant strain of *S. aureus*.

TABLE 5. *Antiresistant staphylococcal activity**

| Organism | Penicillin G | Levopropylcillin | Dextropropylcillin |
|---|---|---|---|
| V-10 | 31 | 2 | 6 |
| V-12 | 36 | 3 | 16 |
| V-18 | 50 | 2 | 12 |
| V-28 | 60 | 4 | 16 |
| V-30 | 54 | 5 | 19 |
| V-32 | 64 | 6 | 12 |
| V-41 | 31 | 3 | 8 |

*Values in μg/ml to inhibit the strains of penicillin-resistant staphylococci. Penicillin prepared from *d*-α-phenoxybutyric acid.

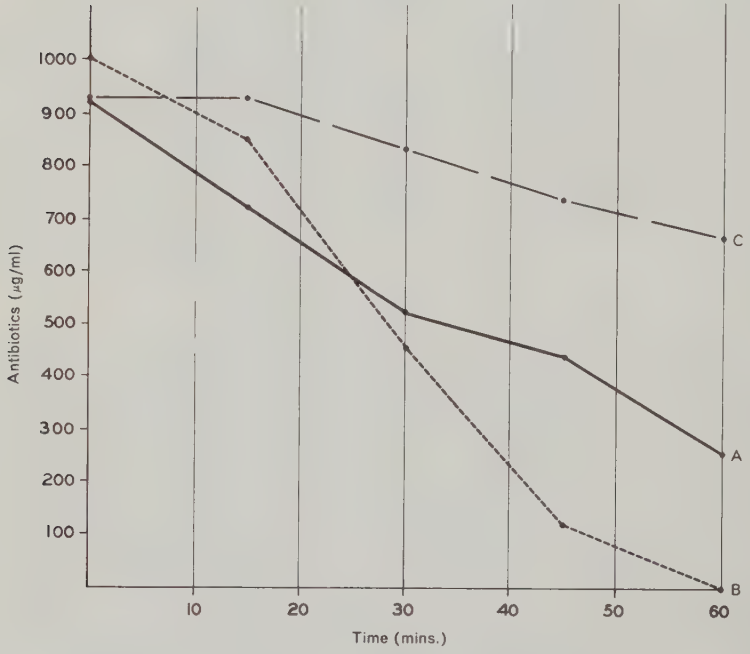


FIG. 2. *Comparison of the time course of loss of antibiotic activity in the presence of staphylococcal penicillinase obtained from crude broth of culture V-41. Symbols:* (A) *penicillin G;* (B) *levopropylcillin;* (C) *dextropropylcillin.*

Figure 3 is a graph of the data obtained when resistant staphylococci were sonically ruptured, the cellular debris centrifuged out at 18,000 × *g*, and the various penicillins added to the penicillinase-containing, clear, supernatant fluid.

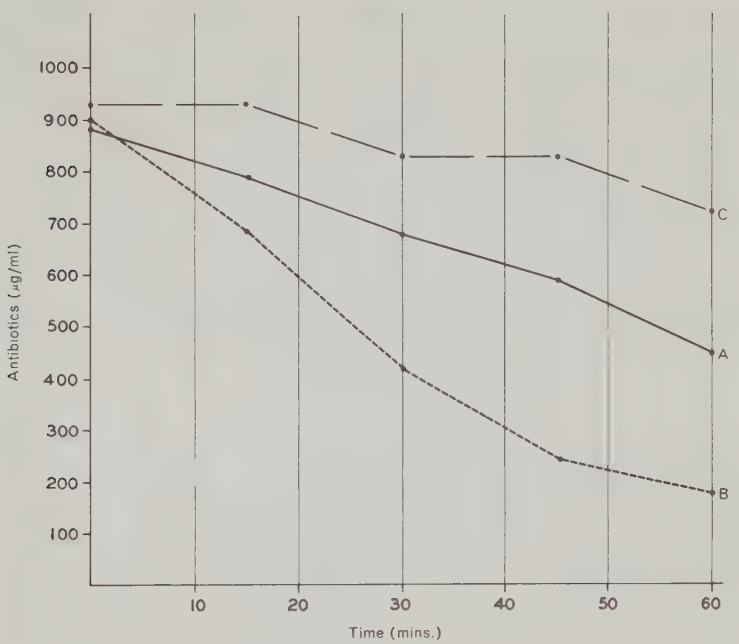


FIG. 3. *Comparison of the time course of loss of antibiotic activity in the presence of staphylococcal penicillinase obtained from sonically ruptured cells of culture V-41. Symbols:* (A) *penicillin G;* (B) *levopropylcillin;* (C) *dextropropylcillin.*

The penicillin prepared from the *d*-α-phenoxybutyric acid, dextropropylcillin, was considerably more stable to staphylococcal penicillinase than was the levopropylcillin, and yet the dextropropylcillin was less active against penicillin-resistant staphylococci than was levopropylcillin. From the experimental data, it appeared that the levopropylcillin was even more sensitive to staphylococcal penicillinase than was penicillin G.

## Discussion

Many publications over the years contain rather strong suggestions that clinically important staphylococci are resistant to penicillin(s) because they are able to elaborate an enzyme called penicillinase, which is capable of destroying the antibiotic activity of the penicillin molecule. Geronimus and Cohen (1957) gave credit to Kirby (1944) for the presentation of evidence that the penicillin resistance of pathogenic staphylococci was due to penicillinase production

by these resistant cells. Williamson et al. (1961) continued along this line, in their explanation of the activity of the isomeric mixture of α-phenoxypropylcillin, when they suggested that the decreased inoculum effect on the activity of this penicillin was due to the increased resistance of this antibiotic to staphylococcal penicillinase. The data of Williamson et al. (1961) were also supported by Jackson and Rao (1961) but these authors noted that perhaps some additional explanation of the apparent activity against resistant staphylococci may be required. A note by Rolinson (1961), concerned with work on α-phenoxypropyl penicillin, leads to the conclusion that he, too, considers penicillinase stability a requirement for antiresistant staphylococcal activity. The case of α-phenoxypropyl penicillin is, then, a paradox because the two isomers seem to have their values mixed. The least penicillinase-stable isomer is the most active against penicillinase-producing resistant staphylococci.

Evidence and suggestions are now appearing in the literature that penicillin may very well have inhibitory action, as yet unexplained (Barber, 1960; Hugo and Russell, 1961; Thatcher and Roberts, 1961). The mechanism of action of levopropylcillin may differ from that of other penicillins in its affinity for another action-site, common to all penicillins, yet quantitatively less important than cell-wall inhibition. Many hypotheses may be advanced, even one that considers the possibility that penicillin-resistant staphylococci may differ in some aspect of their metabolism other than the ability to produce the enzyme, penicillinase.

## Literature Cited

Barber, M. 1960. "Celbenin"-resistant staphylococci. Brit. Med. J. 2:939-940.

Batchelor F. R., F. P. Doyle, J. H. C. Nayler, and G. N. Rolinson. 1959. Synthesis of penicillin: 6-aminopenicillanic acid in penicillin fermentations. Nature 183:257-258.

Bryson, V., and W. Szybalski. 1952. Microbial selection. Science 116:45-46.

Ford, J. H. 1947. Hydroxylamine method of determining penicillins. Ind. Eng. Chem., Anal. Ed. 19:1004-1006.

Geronimus, L. H., and S. Cohen. 1957. Induction of staphylococcal penicillinase. J. Bacteriol. 73:28-34.

Hugo, W. B., and A. D. Russell. 1961. Action of penicillin on *Aerobacter cloacae*. J. Bacteriol. 82:411-417.

Jackson, F. L., and K. K. Rao. 1961. Comparative *in vitro* activities of five penicillins against penicillinase-producing staphylococci. Lancet 22:850-851.

Kirby, W. M. M. 1944. Extraction of a highly potent penicillin inactivator from penicillin-resistant staphylococci. Science 99:452-453.

Rolinson, G. N. 1961. α-Phenoxypropylpenicillin. Lancet 2:941-942.

Thatcher, K. F. J., and E. R. Roberts. 1961. An isotopic study of the action of penicillin on bacterial nitrogen metabolism. Biochim.et Biophys. Acta 49:441-450.

Williamson, G. M., J. K. Morrison, and K. J. Stevens. 1961. A new synthetic penicillin, PA-248. Lancet 2:847-850.

# ABSORPTION, DISTRIBUTION, AND EXCRETION OF *l*-α-PHENOXYPROPYL, *d*-α-PHENOXYPROPYL, *dl*-α-PHENOXYETHYL, AND PHENOXYMETHYL PENICILLINS

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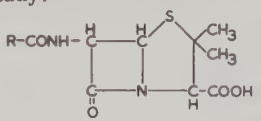
Since penicillin V was first prepared (Behrens et al., 1948), a number of semisynthetic penicillins of the α-phenoxyalkyl series have been prepared and found to display significant biological activity against penicillinase-producing resistant staphylococci. This paper reports a detailed study of the absorption, distribution, and excretion of *l*-α-phenoxypropyl (levopropylcillin), *d*-α-phenoxypropyl (dextropropylcillin), *dl*-α-phenoxyethyl (phenethicillin), and phenoxymethyl (penicillin V) penicillins.

Penicillin V and phenethicillin are the N-phenoxyacetyl and N-α-phenoxypropionyl derivatives, respectively, of 6-amino-penicillanic acid; levopropylcillin and dextropropylcillin are the N-α-phenoxybutyryl derivatives (Fig. 1). The existence of the latter two antibiotics is possible because of the asymmetric carbon atom in the N-acyl group. These chemical differences among the four penicillins are associated with great differences in their pharmacological properties, as presented in this study.




| R-GROUP | |
|---|---|
| PENICILLIN V | $C_6H_5OCH_2-$ |
| PHENETHICILLIN | $C_6H_5OCH(CH_3)-$ |
| PROPYLCILLIN<br>LEVOPROPYLCILLIN<br>DEXTROPROPYLCILLIN | $C_6H_5OCH(C_2H_5)-$ |

FIG. 1. *Structures of levopropylcillin, dextropropylcillin, phenethicillin, and penicillin V.*

## Materials and Methods

*Antibiotics.* Potassium salts of levopropylcillin, dextropropylcillin, phenethicillin (commercial lot M0042, obtained from Bristol-(Myers), and penicillin V were used for these studies. Crystalline preparations of each were given to dogs and rats, either orally in capsules or parenterally dissolved in physiological saline.

*Intravenous injections.* Six healthy female mongrel dogs, weighing between 8.7 and 12.5 kg, were employed. Each dog was given intravenously 5 mg/kg of each of the four antibiotics as a 0.5% solution in saline over a period of 1 min. Blood and urine samples were obtained at intervals and refrigerated until the time of antibiotic assay. Water was given by stomach tube to maintain a urine flow of 1 ml or more per min. Each dog was allowed to rest at least 3 days before the next dose was given.

*Oral administration.* The same six dogs were given orally 10 mg/kg of each of the four antibiotics. Blood and urine samples were taken at intervals and refrigerated.

Thirty-six female Holtzman rats, weighing between 200 and 250 g, were fasted overnight. Nine of these rats were given 25 mg/kg of each of the four antibiotics orally as a 1% solution in saline. Blood samples were taken from the ophthalmic plexus at intervals.

*Renal clearance.* The dogs that received oral administration of the four antibiotics were employed for this study. At the time of antibiotic administration, 0.15 g/kg of creatinine, dissolved in warm saline as a 10% solution, was injected subcutaneously in both front flank regions of each dog. Starting at 45 min, three consecutive urine collections of 15 min duration were obtained by means of a self-retaining catheter. Adequate water was given at the time of creatinine administration to insure a urine flow of 3 ml or more per min during clearance periods. Blood samples were drawn from the jugular vein midway through each period and heparinized. Creatinine concentrations were determined on the cadmium hydroxide plasma filtrates (Fujita and Iwatake, 1931) and on the diluted urine by the alkaline picrate method (Folin and Wu, 1919).

*Plasma binding.* The ultrafiltration method (Coolidge, 1940) was used to separate the filtrate from the plasma protein. About 5 to 8 ml of plasma were pipetted into a dialyzer tubing (diam inflated to 0.75 in.) that was soaked in physiological saline and dried at room temperature. The plasma-filled cellophane sack was placed in a heavy-walled glass tube (5 in. long, 1 in. in diam) about one-half in. from the bottom. The tube was secured with a cork and centrifuged for about 1 hr at 3,000 rev/min. The volume of 0.3 to 0.5 ml of filtrate obtained was used for antibiotic assay. Samples of plasma were simultaneously centrifuged in heavy-walled test tubes covered with metal caps. From the antibiotic concentrations in the plasma

and in the ultrafiltrate, the amount of antibiotics bound to plasma protein was calculated.

*Site of absorption of levopropylcillin.* 1) Dogs:—Sixteen healthy female mongrel dogs, weighing between 8.1 and 15.0 kg, were fasted overnight and anesthetized with 150 mg/kg of phenobarbital. A dose of 10 mg/kg of levopropylcillin as a 1% solution in physiological saline was administered to the lumen of the ligated stomach of four dogs, and to the duodenum, second half of the small intestine, and the colon, respectively, of four other dogs through a mid-abdominal incision, which was closed with sutures. Blood samples were drawn at intervals, and urine samples were collected through a self-retaining catheter.

2) Rats:—Fourteen female Holtzman rats, weighing between 200 and 250 g, were fasted overnight. A mid-abdominal incision was made under light ether anesthesia, and a double ligature was placed at the junction of the stomach and duodenum of all rats. A dose of 25 mg/kg of levopropylcillin as a 1% solution in saline was administered to the stomach of seven rats and to the duodenum of seven other rats. The incision was closed and all rats were allowed to recover from anesthesia. Blood samples were drawn from the ophthalmic plexus at intervals for antibiotic assays.

*Microbiological assay.* Antibiotic activity was determined by an adaptation of the FDA procedure (Kirshbaum et al., 1953), using *Sarcina lutea* in undiluted serum, in serum appropriately diluted with normal serum, and in plasma filtrate and urine appropriately diluted with physiological saline. Normal dog and rat sera were used for setting up the standard curves for the appropriate determinations in serum; physiological saline was used for determinations in plasma filtrate and diluted urine. Each sample was assayed against a sample of the particular antibiotic studied.

*Calculations.* It was felt that geometric means and their standard errors were more appropriate to use than the arithmetic means and their counterparts. Therefore, the former were used throughout this report.

Renal clearance of antibiotic ($C_a$) or of creatinine was calculated from the standard formula. Creatinine clearance was used to measure the glomerular filtration rate.

The concentrations of antibiotic in the serum after intravenous administration decreased in two phases: an initial rapid fall, followed by a slower rate. These two rates of decline can be demonstrated by plotting on semilogarithmic paper. The second slope (b) was calculated by the method of regression lines. The half-life of the antibiotic in serum ($T_{\frac{1}{2}}$) was calculated through the formula:

$$T_{\frac{1}{2}} = \frac{-\frac{1}{2}a}{b}$$

where $a$ represents the supposed serum concentration immediately after completion of the injection if the steady state had been reached instantly and was determined through the formula:

$$a = y - bx$$

Relative volume distribution (RVD) was expressed in ml/g:

$$\text{RVD} = \frac{\text{dosage in mg/kg}}{a}$$

Absolute volume distribution (AVD) was given in liters:

$$\text{AVD} = \text{RVD} \times \text{body wt in kg}$$

Rate of elimination of antibiotic (K) from serum was expressed as per cent per hr:

$$\text{K} = 2.3026b \times 100, \text{ or } \text{K} = \frac{0.693}{\text{T}_{\frac{1}{2}}} \times 100$$

Total clearance of antibiotic ($C_t$) from serum was calculated in ml/min:

$$C_t = \frac{\text{K} \times \text{AVD}}{60}$$

Rate of elimination by the kidney ($K_r$) was calculated as per cent extracted per hr:

$$K_r = \frac{C_a \times 60}{\text{AVD}} \times 100$$

Nonrenal elimination rate ($K_{nr}$) was given as per cent per hr:

$$K_{nr} = \text{K} - K_r$$

The amount of absorption of a drug after oral administration equals the sum of the amounts excreted in the urine, remaining in the body, and metabolized by the tissues. The per cent absorption of these antibiotics in dogs was estimated according to the method of McCance and Madders (1930) by frequent determinations of antibiotics excreted in the urine after both oral and intravenous administrations to the same dog.

## Results

*Antibiotic concentrations in serum after intravenous administration.* Figure 2 shows the calculated regression lines from antibiotic

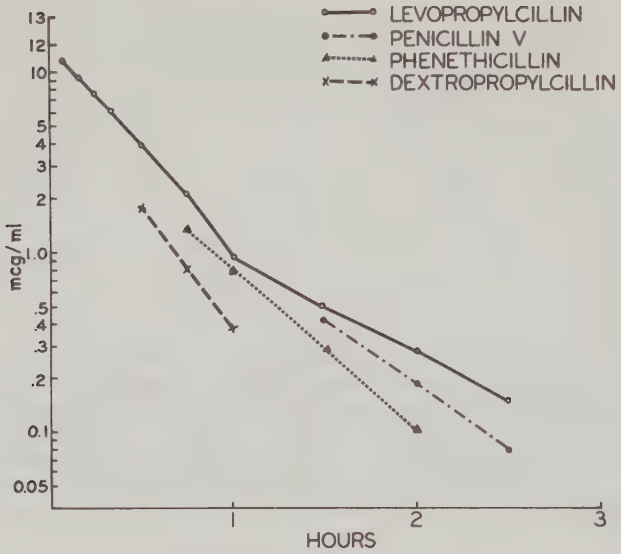



FIG. 2. *Calculated regression lines from serum antibiotic concentrations in dogs after a single intravenous administration of 10 mg/kg of levopropylcillin, dextropropylcillin, phenethicillin, and penicillin V.*

concentrations in sera of six dogs after single intravenous administration of 10 mg/kg of the four antibiotics studied. The initial rapid decline, representing the development of a steady state, was completed within 1 hr for all four antibiotics. The second, slower rate depended solely upon the excretory and metabolic mechanisms. Comparisons of this second rate of decline reveal that the concentrations of levopropylcillin in serum decreased slower than those of the other three antibiotics. The levels of dextropropylcillin fell at the most rapid rate, whereas those of penicillin V and phenethicillin declined at intermediate rates.

*Rates of elimination and half-lives.* Rates of elimination, half-life, and total clearance from serum are summarized in Table 1. Levopropylcillin was eliminated from serum most slowly, with an average rate of 144 ± 12% per hr. Dextropropylcillin showed the most rapid rate, averaging 298 ± 9% per hr, whereas the rates of removal for penicillin V and phenethicillin averaged 162 ± 12 and 201 ± 11% per hr, respectively. Accordingly, levopropylcillin showed the longest serum half-life, averaging 30.7 ± 2.7 min; the half-lives of penicillin V and phenethicillin were intermediate at 25.6 ± 1.9 and 20.7 ± 1.2 min, respectively, whereas that of dextropropylcillin was the shortest, averaging 14.0 ± 0.4 min. The total rates of clearance from serum of these four antibiotics were not significantly different.

TABLE 1. *Disappearance of levopropylcillin, dextropropylcillin, phenethicillin, and penicillin V from serum after a single intravenous administration*

| Penicillins | Serum clearance* (ml/min/$m^2$ ± S.E.) | Rate of elimination (% per hr ± S.E.) | Half-life (min ± S.E.) |
|---|---|---|---|
| Levopropylcillin | 698 ± 109 | 144 ± 12 | 30.7 ± 2.7 |
| Dextropropylcillin | 704 ± 78 | 298 ± 9 | 14.0 ± 0.4 |
| Phenethicillin | 592 ± 66 | 201 ± 11 | 20.7 ± 1.2 |
| Penicillin V | 553 ± 84 | 162 ± 12 | 25.6 ± 1.9 |

*Body surface area calculated by the Meeh-Rubner weight formula (Smith, 1951): $m^2$ (square meter) = 0.10 × wt $(kg)^{0.667}$. S.E. = standard error.

Table 2 gives the renal and nonrenal removal rates for these four antibiotics. The renal removal rates were slowest for levopropylcillin, most rapid for dextropropylcillin, and intermediate for phenethicillin and penicillin V. Correspondingly, the half-lives of the removal rates through kidney were longest for levopropylcillin, shortest for dextropropylcillin, and intermediate for phenethicillin and penicillin V.

TABLE 2. *Renal and nonrenal rates of elimination of levopropylcillin, dextropropylcillin, phenethicillin, and penicillin V*

| Penicillins | Rates of elimination (% per hr ± S.E.) | | Half-life (min ± S.E.) | |
|---|---|---|---|---|
| | Renal | Nonrenal | Renal | Nonrenal |
| Levopropylcillin | 39.2 ± 7.4 | 102.0 ± 9.5 | 106.0 ± 20.1 | 41.0 ± 4.0 |
| Dextropropylcillin | 149.7 ± 17.6 | 130.6 ± 12.3 | 27.7 ± 3.3 | 29.4 ± 2.7 |
| Phenethicillin | 62.3 ± 16.3 | 128.7 ± 14.9 | 66.7 ± 17.4 | 32.3 ± 3.7 |
| Penicillin V | 68.3 ± 17.9 | 78.0 ± 14.9 | 60.9 ± 15.9 | 52.3 ± 10.2 |

The rate of removal through nonrenal mechanisms was slower for levopropylcillin and penicillin V, faster for phenethicillin and dextropropylcillin. The half-lives for these removal rates were longer for the first two antibiotics and shorter for the last two.

*Volumes of distribution.* The values for RVD and AVD are given in Table 3. All antibiotics showed values for RVD greater than for relative blood volumes. Levopropylcillin had the largest RVD, averaging 1.44 ± 0.16 ml/g. This is significantly greater than those for the other three antibiotics. Accordingly, the values for AVD were largest for levopropylcillin, smallest for dextropropylcillin and phenethicillin, and intermediate for penicillin V.

TABLE 3. *Relative (RVD) and absolute (AVD) volume distributions of levopropylcillin, dextropropylcillin, phenethicillin, and penicillin V after a single intravenous administration*

| Penicillins | RVD (ml/g ± S.E.) | AVD (liters ± S.E.) |
|---|---|---|
| Levopropylcillin | 1.44 ± 0.16 | 14.63 ± 1.63 |
| Dextropropylcillin | 0.66 ± 0.09 | 6.69 ± 0.98 |
| Phenethicillin | 0.81 ± 0.13 | 8.63 ± 1.45 |
| Penicillin V | 0.93 ± 0.18 | 10.26 ± 2.27 |

*Plasma protein binding and renal clearances.* Plasma protein binding, renal clearance, and the clearance ratio (antibiotic/creatinine) of each antibiotic are shown in Table 4. The amount of antibiotic bound to plasma protein was about the same for levopropylcillin, phenethicillin, and penicillin V, averaging 52.0 to 55.4%, whereas that of dextropropylcillin was significantly less (43.2%). The adjusted values for renal clearances of all four antibiotics are greater than those of creatinine, resulting in antibiotic/creatinine clearance ratios averaging 2.33 to 3.66. This indicates that these four antibiotics were secreted by the renal tubules.

TABLE 4. *Renal clearance of levopropylcillin, dextropropylcillin, phenethicillin, and penicillin V*

| Penicillins | Total clearance* ($ml/min/m^2$) | Plasma binding (%) | Adjusted clearance ($ml/min/m^2$) | Creatinine clearance ($ml/min/m^2$) | Clearance ratio |
|---|---|---|---|---|---|
| Levopropylcillin | 102.4 ± 13.5 | 53.7 ± 2.2 | 243.4 ± 12.7 | 92.2 ± 6.6 | 2.58 ± 0.22 |
| Dextropropylcillin | 167.0 ± 9.6 | 43.2 ± 2.8 | 305.6 ± 27.3 | 83.8 ± 3.3 | 3.66 ± 0.33 |
| Phenethicillin | 91.8 ± 20.5 | 52.0 ± 7.2 | 200.8 ± 22.7 | 86.5 ± 4.7 | 2.33 ± 0.26 |
| Penicillin V | 116.5 ± 13.0 | 55.4 ± 3.5 | 264.1 ± 36.5 | 81.7 ± 4.9 | 3.26 ± 0.30 |

*Body surface calculated by the Meeh-Rubner weight formula (Smith, 1951): $m^2$ (square meter) = $0.10 \times wt\ (kg)^{0.667}$.

*Urinary recovery and oral absorption:* Urinary recovery of these antibiotics after intravenous and oral administration is summarized in Table 5. After intravenous administration, the values for urinary recovery within 6 hr were largest for penicillin V, averaging 39.3 ± 4.6% of the dose. Those values for levopropylcillin, dextropropylcillin, and phenethicillin averaged 29.8 ± 2.5%, 25.8 ± 2.8%, and 24.7 ± 1.0% of the dose, respectively. On the other hand, the urinary recovery of levopropylcillin after oral administration to the same group of six dogs averaged 23.6 ± 2.0% of the dose. This is significantly higher than those of penicillin V, phenethicillin, and dextropropylcillin following the same route, namely, 11.6 ± 2.3%, 10.1 ± 1.2%, and 10.0 ± 1.2% of the dose, respectively.

TABLE 5. *Urinary antibiotic recoveries after oral or intravenous administration of levopropylcillin, dextropropylcillin, phenethicillin, and penicillin V*

| Penicillins | Dose ± S.E. (%) | |
|---|---|---|
| | Intravenous | Oral |
| Levopropylcillin | 29.8 ± 2.5 | 23.6 ± 2.0 |
| Dextropropylcillin | 25.8 ± 2.8 | 10.0 ± 1.2 |
| Phenethicillin | 24.7 ± 1.0 | 10.1 ± 1.2 |
| Penicillin V | 39.3 ± 4.6 | 11.6 ± 2.3 |

Figure 3 shows the antibiotic concentrations in the sera of dogs after oral administration of 10 mg/kg of these antibiotics. Levopropylcillin produced the highest serum concentrations, reaching a peak level of 6.35 μg/ml in about 1 hr. Significant levels of activity were detected 5 hr after administration. Phenethicillin and penicillin V produced peak levels of 3.80 and 2.88 μg/ml, respectively, at about the same time, whereas a peak level of 2.69 μg/ml was reached 30 min earlier after administration of dextropropylcillin.

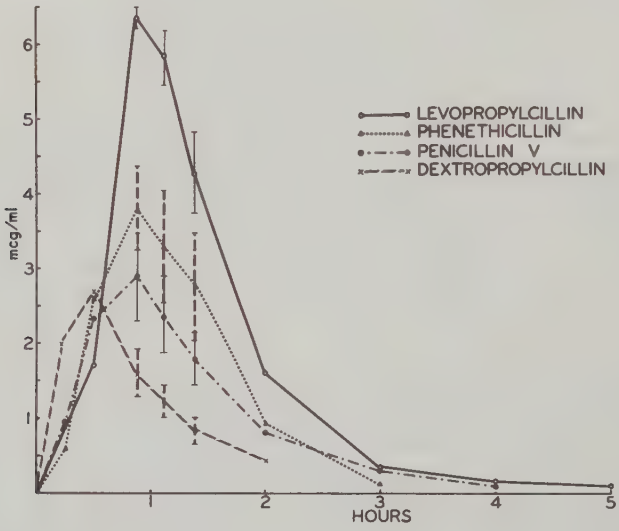



FIG. 3. *Serum antibiotic concentrations in dogs after oral administration of 10 mg/kg of levopropylcillin, dextropropylcillin, phenethicillin, and penicillin V.*

The values for the calculated per cent of absorption after oral administration of these antibiotics are presented in Table 6. A total

TABLE 6. *Calculated cumulative absorption after oral administration of levopropylcillin, dextropropylcillin, phenethicillin, and penicillin V*

| Penicillins | Dose ± S.E. (%) | | | |
|---|---|---|---|---|
| | 1 hr | 2 hr | 4 hr | 6 hr |
| Levopropylcillin | 54.6 ± 3.8 | 79.7 ± 5.5 | 82.5 ± 6.5 | 83.2 ± 6.7 |
| Dextropropylcillin | 31.8 ± 3.5 | 37.8 ± 3.1 | 38.7 ± 3.3 | 38.8 ± 3.4 |
| Phenethicillin | 29.3 ± 4.1 | 40.5 ± 3.6 | 42.6 ± 3.3 | 42.8 ± 3.3 |
| Penicillin V | 25.2 ± 4.7 | 30.0 ± 5.1 | 33.2 ± 4.5 | 33.3 ± 4.5 |

of 83.2 ± 6.7% of the orally administered levopropylcillin was absorbed in 6 hr. The values for those of phenethicillin, dextropropylcillin, and penicillin V averaged 42.8 ± 3.3%, 38.8 ± 3.4%, and 33.3 ± 4.5% of the oral dose, respectively. Two-thirds of these totals were absorbed during the first hour after administration, and nearly 99% within 4 hr.

As shown in Fig. 4, levopropylcillin also produced the best serum concentrations in rats after oral administration of 25 mg/kg. The four antibiotics reached peak levels in serum within 30 min.

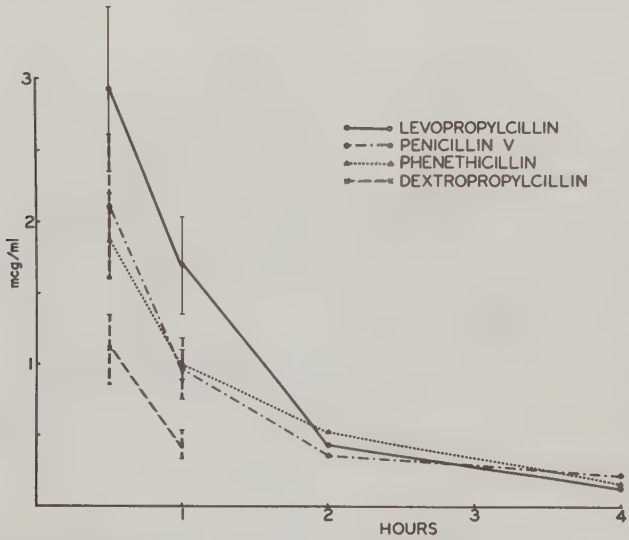



FIG. 4. *Serum antibiotic concentrations in rats after oral administration of 25 mg/kg of levopropylcillin, dextropropylcillin, phenethicillin, and penicillin V.*

*Site of absorption of levopropylcillin.* Figure 5 illustrates the antibiotic concentrations in serum of dogs after administration of 10 mg/kg of levopropylcillin to different sites of the gastrointestinal

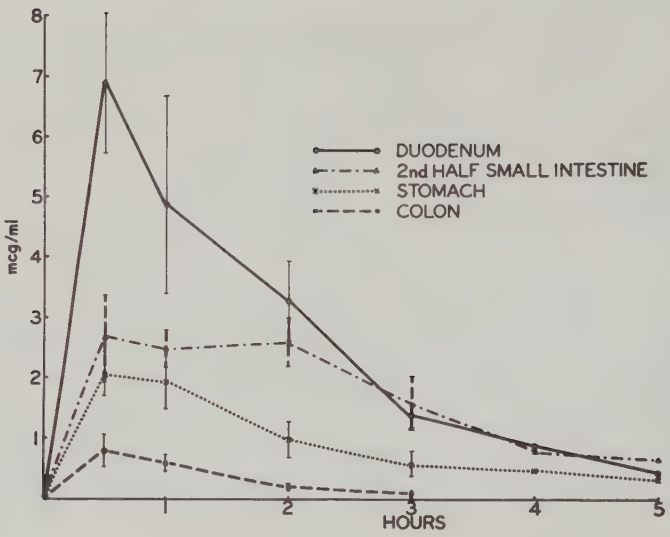



FIG. 5. *Serum antibiotic concentrations in dogs after administration of 10 mg/kg of levopropylcillin to different sites of the gastrointestinal tract.*

tract. The highest serum concentrations were produced when levopropylcillin was administered to the duodenum. When it was administered to the second half of the small intestine, stomach, or colon, significant absorption also took place (in decreasing order). The amounts of urinary recovery of this antibiotic were in close agreement with the serum concentrations. These dogs excreted 20.8, 13.5, 6.8, and 1.5% of the dose in 5 hr when levopropylcillin was administered to the duodenum, second half of the small intestine, stomach, or colon, respectively.

Better absorption of levopropylcillin was also observed after administration to the duodenum of rats (Fig. 6). When levopropylcillin was administered to the stomach, the antibiotic concentrations in serum were much lower.

## Discussion

Whether the serum level of an antibiotic is the determining factor for its therapeutic effect is controversial. An antibiotic that produces high serum levels often does not easily reach the tissues where the action apparently takes place. Thus, it is reasonable to believe that the more the antibiotic penetrates to the tissues, the

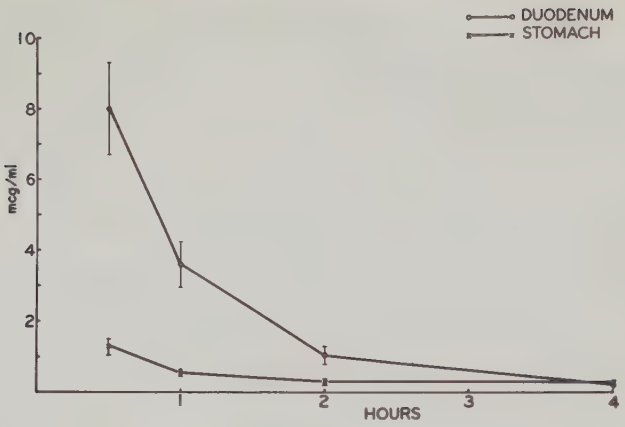



FIG. 6. *Serum antibiotic concentrations in rats after administration of 25 mg/kg of levopropylcillin to different sites of the gastrointestinal tract.*

better its therapeutic effect. Spitzy and Hitzenberger (1958) discussed the use of distribution volume as the best indication of penetration of the antibiotic to the tissues. Their data demonstrated that, after intravenous administration of various antibiotics, high concentration in serum resulted in a low distribution volume, and vice versa.

In the present study, all four antibiotics produced volumes of distribution larger than the total blood volumes after intravenous administration (Table 3). This indicates the penetration of these antibiotics beyond the blood pool. The values for RVD of levopropylcillin and penicillin V were 1.44 ± 0.16 ml/g and 0.93 ± 0.18 ml/g, respectively. It would imply that these antibiotics are concentrated in the tissues. Although tissue distribution of these antibiotics has not been studied, high concentrations of other antibiotics in various tissues were reported (Lee, Anderson, and Chen, 1957; Anderson et al., 1959). The values for RVD of penicillin V in these dogs were slightly higher but not significantly different from those reported for human subjects (Spitzy and Hitzenberger, 1958; Kunin, 1961).

Serum concentration of levopropylcillin, after a single intravenous administration, decreased most slowly among these antibiotics (Fig. 2). Dextropropylcillin in the serum declined most rapidly, whereas the rates of decline of the other two antibiotics were intermediate. This is best understood when the rates are expressed as rates of elimination and as half-lives in the serum (Table 1). Levopropylcillin was eliminated from serum at a rate of 144 ± 12% per hr and had an average half-life of 30.7 ± 2.7 min, whereas the removal rate of dextropropylcillin was 298 ± 9% per hr and the half-life

averaged 14.0 ± 0.4 min. The values for half-life and rate of elimination of penicillin V from serum in human subjects (Spitzy and Hitzenberger, 1958; Kunin, 1961) do not differ significantly from those in dogs in the present study.

The percentage of levopropylcillin, phenethicillin, and penicillin V bound to plasma protein averaged 52.0 to 55.4. This is significantly higher than the penicillin V value, obtained by use of the dialysis technique (Anderson et al., 1956). This may be expected from the fact that different procedures were used. Dextropropylcillin is bound to plasma to a lesser degree (43.2%).

The renal clearance ratios (antibiotic/creatinine) were fairly distinct for each of the four antibiotics. They varied from an average of 2.33 for phenethicillin to 3.66 for dextropropylcillin, indicating that all antibiotics were secreted by the renal tubules. The value for penicillin V is not significantly different from that reported previously (Anderson et al., 1956).

Urinary recoveries after intravenous administration of all antibiotics were less then half of the dose. These findings can be better understood by calculations of rate of removal by nonrenal mechanisms (Table 2). Each antibiotic was found to be removed at a faster rate by nonrenal routes than by the kidney, except dextropropylcillin, which was removed rapidly by both renal and nonrenal mechanisms.

Levopropylcillin produced better serum concentrations after oral administration to both dogs (Fig. 3) and rats (Fig. 4) than comparable doses of dextropropylcillin, phenethicillin, and penicillin V. A total of 23.6 ± 2.0% of the orally administered levopropylcillin was excreted in the urine of dogs within 6 hr, as compared with half of this amount for the other three antibiotics (Table 5). A direct determination for oral absorption of a drug is lacking. Nevertheless, calculations can be made, based upon urinary excretion after both oral and intravenous administration to the same individual (McCance and Madders, 1930). In the present study, a group of six dogs absorbed an average of 83.2 ± 6.7% of the orally administered levopropylcillin. This value is far larger than those obtained after oral administration of dextropropylcillin, phenethicillin, and penicillin V to the same group of dogs. Absorption of these antibiotics took place within 1 hr after administration and was nearly completed within 4 hr.

The potassium salt of penicillin V was found to be absorbed mainly in the stomach of dogs and to a lesser degree in the intestine (Lee et al., 1958). Levopropylcillin produced higher serum concentrations after administration to the duodenum than after administration to the second half of the small intestine, the stomach, or the colon. This difference in site of absorption may be related to differences in chemical and physical properties.

## Acknowledgments

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## Literature Cited

Anderson, R. C., C. C. Lee, H. M. Worth, and K. K. Chen. 1956. Pharmacologic and toxicologic studies with penicillin V. Antibiotics Ann. 1955-1956, p. 540-548.

Anderson, R. C., C. C. Lee, H. M. Worth, and P. N. Harris. 1959. Pharmacology and toxicology of propionyl erythromycin ester lauryl sulfate. J. Am. Pharm. Assoc., Sci. Ed. **48**:623-628.

Behrens, O. K., J. Corse, J. P. Edwards, L. Garrison, R. G. Jones, Q. F. Soper, F. R. Van Abeele, and C. W. Whitehead. 1948. Biosynthesis of penicillin. IV. New crystalline biosynthetic penicillins. J. Biol. Chem. **175**:793-809.

Coolidge, T. B. 1940. A simple ultra-filtration apparatus. J. Biol. Chem. **135**:541-542.

Folin, O., and H. Wu. 1919. A system of blood analysis. J. Biol. Chem. **38**:81-110.

Fujita, A., and D. Iwatake. 1931. Bestimmung des echten Blutzuckers ohne Hefe. Biochem. Z. **242**:43-60.

Kirshbaum, A., F. W. Bowman, D. M. Wintermere, and E. R. Friedman. 1953. A cup-plate method for the determination of erythromycin concentrations in serum and other body fluids. Antibiotics & Chemotherapy **3**:537-539.

Kunin, C. M. 1961. Serum binding, distribution and excretion of four penicillin analogues following intravenous injection in man. Proc. Soc. Exptl. Biol. Med. **107**:337-341.

Lee, C. C., R. C. Anderson, and K. K. Chen. 1957. Vancomycin, a new antibiotic. V. Distribution, excretion and renal clearance. Antibiotics Ann. 1956-1957, p. 82-89.

Lee, C. C., R. O. Froman, R. C. Anderson, and K. K. Chen. 1958. Gastric and intestinal absorption of penicillin V potassium and the free acid. Antibiotics & Chemotherapy **8**:354-360.

McCance, R. A., and K. Madders. 1930. The comparative rates of absorption of sugars from the human subjects. Biochem. J. **24**:795-804.

Smith, H. W. 1951. The kidney structure and function in health and disease, p. 562. Oxford University Press, New York.

Spitzy, K. H., and G. Hitzenberger. 1958. The distribution volume of some antibiotics. Antibiotics Ann. 1957-1958, p. 996-1003.

# COMPARISON OF TWO NEW DERIVATIVES OF 6-AMINOPENICILLANIC ACID WITH METHICILLIN

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Since the isolation of 6-aminopenicillanic acid by Batchelor et al. (1959), considerable effort has been expended to synthesize new penicillins which would be resistant to the activity of the penicillinase of staphylococci. Such compounds have been anticipated to provide effective antistaphylococcal action against most clinical strains, with no greater toxicity than noted for benzyl penicillin G. Methicillin and, more recently, 5-methyl-3-phenyl-4-isoxazolyl-penicillin have been introduced, after early studies indicated usefulness against large numbers of staphylococci.

Recent work by chemists of the Wyeth Research Division, Wyeth Laboratories, has resulted in the synthesis of 6-(2-ethoxy-1-naphthamido)penicillanic acid (Wy 3277) and 6-(2-methoxy-1-naphthamido) penicillanic acid (Wy 3206). Resistance to degradation by penicillinase has been shown for these compounds (Rosenman and Warren, 1961). In our laboratory, early in vitro tests against clinical isolates gave encouragement to pharmacological trials in man. This report will present data suggesting clinical usefulness of one or perhaps both of these compounds.

## Materials and Methods

In vitro-sensitivity testing was carried out with the replica inoculation, plate dilution method, previously described by Foltz, Steers, and Graves (1959) from this laboratory. The penicillins were incorporated in various dilutions in Trypticase soy agar plates with 5% whole blood added.

Pure cultures of fresh clinical isolates of staphylococci and streptococci, other than group A, were obtained from the bacteriology

laboratory for susceptibility testing. Twofold serial dilutions of the penicillins were made from 50 to 0.3 μg/ml in the blood agar medium. Inoculation of media was accomplished with a suspension of organisms in brain heart infusion broth; these organisms were in the stage of active division. Any visible growth or colony formation, after 16 hr of incubation on the antibiotic-containing media, was interpreted as resistance.

Subjects for the pharmacological studies were healthy adult males selected from a prison population. In a crossover study, five men were injected on each test day with 500 mg of either methicillin or one of the naphthamido derivatives. The methicillin was administered in a volume of 1 ml and Wy 3277 and Wy 3206 were administered in a 2-ml volume.

Sera, obtained by vena puncture, were harvested from appropriate blood samples. These sera plus samples of urine were frozen at once, and stored until bio-assay was accomplished.

Bio-assay of serum and urine samples for penicillin content was performed with a modified cup-plate method; *Sarcina lutea* was employed as the test organism. Serum samples were diluted with pooled human serum, when necessary, and all assays were read against standard curves obtained by diluting the standard with pooled human serum. The urine samples were diluted in phosphate buffer solution (pH 6.0) and compared with a standard curve made of standard dilutions in a similar phosphate buffer solution.

## Results

The susceptibility of 186 clinical isolates of staphylococci to Wy 3277 is shown in Table 1. In comparison with methicillin and benzyl penicillin G, it accounted for the inhibition of a greater number of isolates at each concentration except at 25 and 50 μg/ml, where

TABLE 1. *In vitro susceptibility of clinical isolates to Wy 3277, benzyl penicillin, and methicillin*

| Antibiotic | Concentrations in μg/ml | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | 50 | 25 | 12 | 6 | 3 | 1.5 | 0.6 | 0.3 |
| *Staphylococci (186 strains)* | | | | | | | | |
| Penicillin G | 73* | 68 | 65 | 60 | 58 | 54 | 44 | 42 |
| Methicillin | 100 | 100 | 97 | 97 | 94 | 63 | 8 | 2 |
| Wy 3277 | 100 | 100 | 100 | 100 | 99 | 93 | 90 | 80 |
| *Streptococci (60 strains)* | | | | | | | | |
| Penicillin G | 100 | 100 | 100 | 100 | 100 | 78 | 40 | 40 |
| Methicillin | 100 | 88 | 42 | 38 | 38 | 38 | 32 | 23 |
| Wy 3277 | 100 | 100 | 93 | 83 | 62 | 42 | 38 | 38 |

*Inhibition (%) in plate-dilution test.

an equal number of organisms was inhibited by methicillin. At low concentrations (0.6 and 0.3 μg/ml), more than ten times as many strains were inhibited by Wy 3277 as by methicillin, and considerably more isolates were susceptible to Wy 3277 than to penicillin G at these same low concentrations.

Against streptococci, Wy 3277 was slightly less effective than penicillin G; this was observed for moderately resistant strains showing all three types of hemolysis. Wy 3277 was found to inhibit more strains than methicillin except at a concentration of 50 μg/ml. More than 80% of all strains tested were inhibited by 6 μg/ml of Wy 3277.

Wy 3206 was studied at a later date against 226 additional strains of staphylococci (Table 2). This compound, in comparison with penicillin G, phenethicillin, and methicillin, demonstrated superiority in numbers of isolates inhibited at all concentrations, except at 0.3 μg/ml where it was less active than phenethicillin and penicillin G. In the higher concentrations (25 μg/ml or greater), there was no difference in activity between methicillin and Wy 3206, but in the intermediate to lower portions of the dilution series, the activity of Wy 3206 was greater than that of methicillin.

TABLE 2. *In vitro susceptibility of clinical isolates to Wy 3206 and other penicillins*

| Antibiotic | Concentration in μg/ml | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | 50 | 25 | 12 | 6 | 3 | 1.5 | 0.6 | 0.3 |
| *Staphylococci (226 strains)* | | | | | | | | |
| Penicillin G | 68* | 65 | 61 | 57 | 52 | 44 | 39 | 35 |
| Phenethicillin | 77 | 71 | 69 | 67 | 63 | 57 | 53 | 45 |
| Methicillin | 99 | 99 | 93 | 93 | 91 | 54 | 7 | <1 |
| Wy 3206 | 99 | 99 | 99 | 99 | 95 | 90 | 62 | 31 |
| *Streptococci (56 strains)* | | | | | | | | |
| Penicillin G | 100 | 100 | 98 | 95 | 95 | 66 | 5 | 4 |
| Phenethicillin | 100 | 96 | 96 | 95 | 71 | 5 | 5 | 5 |
| Methicillin | 96 | 96 | 7 | 5 | 5 | 5 | 2 | 0 |
| Wy 3206 | 96 | 93 | 91 | 88 | 47 | 7 | 5 | 4 |

*Inhibition (%) in plate-dilution test.

The inhibitory activity of Wy 3206 against streptococci was considerably less than penicillin G in a comparison of the MIC's for 56 strains, but was much better than the activity of methicillin. It proved to be less effective than phenethicillin in concentrations of 1.5 μg/ml or greater.

The serum concentrations, resulting from 0.5-g intramuscular injections of Wy 3277 and methicillin in ten men, are shown in Table 3. Relatively high and prompt absorption for both of these agents is reflected in the high serum concentrations. Both agents provided peak concentrations at 0.5 hr. The penicillemia was better maintained

by Wy 3277 through 6 hr, when all subjects showed assayable concentrations; at 8 hr, only four subjects had concentrations in the assayable range. The serum concentrations were higher for methicillin during the first hour postinjection, but higher for Wy 3277 thereafter.

TABLE 3. *Comparison of serum concentrations after intramuscular injection of Wy 3277 and methicillin*

| Subject | Time in hours | | | | | | |
|---|---|---|---|---|---|---|---|
| | 0 | 0.5 | 1 | 2 | 4 | 6 | 8 |
| *Wy 3277** | | | | | | | |
| 1 | 0 | 6.75 | 5.75 | 4.10 | 2.00 | .46 | .14 |
| 2 | 0 | 4.30 | 4.65 | 5.40 | .88 | .33 | .11 |
| 3 | 0 | 4.65 | 4.55 | 4.30 | .70 | .23 | <.10 |
| 4 | 0 | 5.00 | 4.50 | 3.50 | .79 | .26 | <.10 |
| 5 | 0 | 7.50 | 4.75 | 3.30 | .50 | .11 | <.10 |
| 6 | 0 | 10.40 | 4.95 | 2.70 | .44 | .115 | <.10 |
| 7 | 0 | 8.80 | 8.20 | 7.40 | 1.35 | .34 | <.10 |
| 8 | 0 | 9.60 | 8.00 | 5.80 | 1.90 | .61 | .32 |
| 9 | 0 | 17.00 | 8.80 | 4.70 | .78 | .23 | <.10 |
| 10 | 0 | 5.25 | 4.85 | 4.40 | .92 | .39 | .17 |
| Avg. | | 7.93 | 5.90 | 4.56 | 1.03 | .31 | .07 |
| *Methicillin** | | | | | | | |
| 1 | 0 | 9.50 | 9.90 | 5.00 | .72 | <.30 | <.30 |
| 2 | 0 | 9.00 | 8.40 | 3.20 | .67 | <.30 | <.30 |
| 3 | 0 | 12.25 | 8.10 | 1.95 | .38 | <.30 | <.30 |
| 4 | 0 | 9.50 | 8.10 | 4.10 | .80 | <.30 | <.30 |
| 5 | 0 | 8.45 | 7.40 | 1.55 | <.30 | <.30 | <.30 |
| 6 | 0 | 11.50 | 8.90 | 2.50 | .73 | <.37 | <.37 |
| 7 | 0 | 8.30 | 10.30 | 7.50 | 1.20 | <.37 | <.37 |
| 8 | 0 | 16.50 | 12.50 | 4.60 | .58 | <.37 | <.37 |
| 9 | 0 | 16.00 | 14.00 | 4.20 | .50 | <.37 | <.37 |
| 10 | 0 | 11.00 | 10.30 | 6.10 | .93 | <.37 | <.37 |
| Avg. | | 11.20 | 9.79 | 4.07 | .65 | <.30 | <.30 |

*Dosage was 500 mg for each agent. Results are expressed as μg/ml of sodium salt-unit weight. Potency of standards: Wy 3277 = 950 μg/mg; methicillin = 937 μg/mg.

The average urinary recovery for eight of the men shown in Table 3 is presented in Table 4. Two subjects failed to complete

TABLE 4. *Average urinary recovery of penicillins in eight subjects*

| Penicillin | Intervals | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | 0 to 3 Hr | | 3 to 6 Hr | | 6 to 12 Hr | | Total | |
| | μg | % | μg | % | μg | % | μg | % |
| Wy 3277 | 193,262 | 38.6 | 46,520 | 9.3 | 5341 | 1.1 | 245,123 | 49.0 |
| Methicillin | 301,300 | 60.3 | 32,560 | 6.5 | 6190 | 1.2 | 340,049 | 68.0 |

urine collections and could not be included in the results. It appears that methicillin is recovered to a greater degree and more rapidly than Wy 3277, but in these trials, almost 50% of the injected dose of Wy 3277 was recovered in the first 12 hr. These differences in recovery of the active penicillins are quite compatible with the extent and duration of penicillemia.

Similar pharmacological comparisons of Wy 3206 and methicillin in another ten subjects are shown in Table 5. One man was not available for the injection of Wy 3206 in the crossover study. Average data for the same nine men show that methicillin resulted in higher peak concentrations and longer duration of penicillemia in this trial than did Wy 3206. Peak concentrations were generally observed in the first half hour for both agents, but two subjects showed some delay in absorption, with peak concentrations in their sera at 1 hr for both preparations.

TABLE 5. *Serum concentrations of Wy 3206 and methicillin after intramuscular injection*

| | Time in hours | | | | | | |
|---|---|---|---|---|---|---|---|
| Subject | 0 | 0.5 | 1 | 2 | 4 | 6 | 8 |
| *Wy 3206** | | | | | | | |
| 1† | – | – | – | – | – | – | – |
| 2 | 0 | 3.50 | 4.00 | 2.10 | .51 | .17 | <.15 |
| 3 | 0 | 7.00 | 3.70 | 1.35 | .23 | <.15 | <.15 |
| 4 | 0 | 5.50 | 3.10 | .45 | .42 | <.15 | <.15 |
| 5 | 0 | 4.80 | 3.90 | 1.65 | .37 | <.15 | <.15 |
| 6 | 0 | 3.90 | 3.50 | 1.40 | .23 | <.15 | <.15 |
| 7 | 0 | 7.50 | 3.60 | 1.10 | .28 | <.15 | <.15 |
| 8 | 0 | 2.80 | 3.50 | 2.00 | .55 | .22 | <.15 |
| 9 | 0 | 8.00 | 5.50 | 2.15 | .31 | <.15 | <.15 |
| 10 | 0 | 9.20 | 3.70 | 1.30 | .30 | <.15 | <.15 |
| Avg. (9 men) | | 5.80 | 3.80 | 1.56 | .36 | .04 | .15 |
| *Methicillin** | | | | | | | |
| 1 | 0 | 10.00 | 8.80 | 4.50 | .80 | .15 | <.15 |
| 2 | 0 | 6.80 | 7.00 | 3.10 | .63 | <.15 | <.15 |
| 3 | 0 | 10.00 | 5.50 | 2.20 | .61 | .19 | <.15 |
| 4 | 0 | 8.80 | 6.80 | 2.40 | .53 | <.15 | <.15 |
| 5 | 0 | 5.30 | 5.50 | 3.80 | .87 | .25 | <.15 |
| 6 | 0 | 7.50 | 5.50 | 2.30 | .58 | <.15 | <.15 |
| 7 | 0 | 6.40 | 5.00 | 2.65 | .51 | .21 | <.15 |
| 8 | 0 | 8.50 | 6.30 | 3.30 | .58 | .25 | <.15 |
| 9 | 0 | 8.00 | 7.30 | 4.10 | .66 | .15 | <.15 |
| 10 | 0 | 11.00 | 7.50 | 4.00 | .85 | .31 | <.15 |
| Avg. (9 men) | | 8.03 | 6.26 | 3.10 | .65 | .15 | .15 |

*Dosage was 500 mg for each agent. Results are expressed as μg/ml of sodium salt-unit weight. Potency of standards: Wy 3206 = 1.004 μg/mg; methicillin = 937 μg/mg.

†This patient was not available for injection of Wy 3206.

TAB.E 6. *Average urinary recovery of penicillins in nine subjects*

| Penicillin | Intervals | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | 0 to 3 Hr | | 3 to 6 Hr | | 6 to 12 Hr | | Total | |
| | μg | % | μg | % | μg | % | μg | % |
| Wy 3206 | 106,252 | 21.3 | 26,612 | 5.3 | 8,063 | 1.6 | 140,927 | 28.2 |
| Methicillin | 270,986 | 54.2 | 48,390 | 9.7 | 11,886 | 2.4 | 331,262 | 66.3 |

The average urinary recoveries for the same nine subjects, who were available for comparison of Wy 3206 and methicillin, is shown in Table 6. Less than 30% of the administered dose of Wy 3206 was recovered in the urine and an excess of 60% of the methicillin was excreted in active form.

## Discussion

The synthesis of 6-(2-ethoxy-1-naphthamido) and of 6-(2-methoxy-1-naphthamido) penicillanic acids has contributed two more penicillins, found to be resistant to the activity of staphylococcal penicillinase. Hence, both of these agents were expected to prove effective in inhibiting most clinical isolates of staphylococci which show major resistance to benzyl, phenoxymethyl, or phenoxyethylpenicillins.

The in vitro studies in our laboratory suggested that both the ethoxy and methoxy derivatives of naphthamido penicillanic acid were effective against most staphylococci in relatively low concentrations. In the comparisons, both derivatives showed wider effective inhibition than methicillin. Methicillin has proven in the past to be more effective against staphylococci found to be resistant to penicillin G. Our data confirm the relationship of this older pair of penicillins, although clearly indicating superiority for the antistaphylococcal activity of the naphthamido penicillins over benzyl penicillin and methicillin. Against streptococci, both derivatives proved to have activity, but somewhat less than that of benzyl penicillin and greater than that of methicillin.

On the basis of these in vitro results the naphthamido penicillins seemed qualified for further clinical trials. Although no direct comparison of these two compounds was carried out, there appeared to be relatively little difference between their antistaphylococcal activity, but the 2-ethoxy derivative (Wy 3277) appeared to be somewhat more active in lower concentrations against streptococci.

Pharmacological studies in man were performed as soon as the penicillins were made available and cleared for human trials. Wy 3277 was available first and preceded the trials of Wy 3206.

Both the 2-methoxy and 2-ethoxy derivatives of naphthamido penicillanic acid yielded maximal serum concentrations at 0.5 and 1 hr, which were approximately 70 to 60% of those obtained in the same subjects with methicillin. The duration of effective penicillemia was greater with Wy 3277 (2-ethoxy) than with Wy 3206; at 2 hr postinjection, and, continuing through 6 hr, this compound gave higher average concentrations in the serum than observed for methicillin. On this basis, Wy 3277 would appear to be the better of the two compounds.

Plotting these serum concentration data suggests that the half-life of Wy 3277 in the blood was slightly greater than 1.5 hr. This is approximately the half-life period of methicillin in these two study groups. However, the slope of disappearance from the blood is less steep, with a slightly lower peak and a longer duration of penicillemia, than noted for methicillin. The half-life for Wy 3206 was approximately 75 to 80 min, with a disappearance slope of the same pattern as methicillin.

Urinary recovery data also confirm the fact that Wy 3277 is excreted slower than methicillin, although total recovery is considerably less than that for methicillin (49 and 68%, respectively). Wy 3206 was recovered to a much smaller extent (28%) in another group of subjects which showed recovery of methicillin in the same range as above (66%).

It therefore appears that 6-(2-ethoxy-1-naphthamido) penicillanic acid is superior to the 2-methoxy derivatives on both direct observation of in vitro antibacterial activity and in pharmacological measurement. No direct comparative data are available to support this assumption. It would appear that maximally effective inhibitory concentrations of Wy 3277 can result after a 0.5 g dose for at least 2 hr, although concentrations capable of inhibiting 75% of staphylococcal strains tested persisted for 4 to 5 hr. The serum concentration/MIC ratio is more favorable for Wy 3277 than for methicillin, in the dosage trial described. Additional serum-inhibitory titer tests are indicated to prove these assumptions.

Comparison of subjective pain responses in these individuals following intramuscular injection proved all three preparations capable of producing mild, immediate pain, and slightly more intense delayed pain (1 to 2 hr) in some subjects. No clear-cut pattern of superiority emerged from these subjective responses.

## Acknowledgments

The authors wish to express appreciation for the grant-in-aid for these studies by Wyeth Laboratories, Inc., and the cooperation of Edward Steers, Department of Bacteriology, William Pepper Laboratory, Hospital of the University of Pennsylvania, for supplying clinical subcultures.

## Literature Cited

Batchelor, F. R., F. P. Doyle, J. H. C. Naylor, and G. N. Rolinson. 1959. Synthesis of penicillin: 6 amino penicillanic acid in penicillin fermentations. Nature **183**:257.

Foltz, E. L., E. Steers, B. S. Graves, and H. J. Suriano. Initial comparative study of susceptibility tests as performed with a replicating device. Antibiotics Ann. 1959-1960, p. 596-603.

Rosenman, S. B., and G. H. Warren. 1962. In vitro evaluation of semisynthetic penicillins Wy-3206 and Wy-3277. Antimicrobial Agents and Chemotherapy - 1961, p. 611-619.

# EFFECTS OF SIDE CHAIN CONFIGURATION ON THE ACTIVITY OF CERTAIN SEMISYNTHETIC PENICILLINS

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Since the discovery that 6-aminopenicillanic acid can be obtained by fermentation (Batchelor et al., 1959) or by enzymatic hydrolysis of natural penicillins (Claridge, Gourevitch, and Lein, 1960; Rolinson et al., 1960; Kaufman and Bauer, 1960), a large number of semisynthetic penicillins have been prepared by acylation of the parent compound (Perron et al., 1960*a*). When the acid used in such an acylation contains an asymmetric carbon atom, diastereoisomeric forms of the penicillin are obtained.

In a few cases, the enantiomorphic forms of the acids have been resolved and both diastereoisomeric penicillins have been prepared (Perron et al., 1960*b*). In this paper, we wish to compare the antibacterial activities of the diastereoisomeric forms of penicillins prepared from D- and L-α-phenoxypropionic, α-aminophenylacetic, and α-hydroxyphenylacetic acids and relate them to structural features of the molecules.

## Materials and Methods

Penicillins studied in this paper were prepared from resolved acids by the members of the organic chemistry department of Bristol Laboratories. Penicillins derived from the phenoxypropionic and hydroxyphenylacetic acids were prepared as potassium salts while those derived from aminophenylacetic acids were used as free acids. The designation D and L refers to the Fischer-Rosanoff convention (Hudson, 1948).

Minimal inhibitory concentrations were determined by the standard twofold serial-dilution technique in heart infusion broth or in heart infusion broth mixed 1:1 with pooled-human serum in the case of *Diplococcus pneumoniae* and *Streptococcus pyogenes* organisms. A $10^4$ dilution of an overnight culture was used as an inoculum.

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## Results

As can be seen in Table 1, the L diastereoisomer of the phenoxyethyl penicillin (phenethicillin) is more active than the D isomer. The reverse is true of the other penicillins. The D isomers of α-aminobenzyl and α-hydroxybenzyl penicillins are more active than the L isomers.

TABLE 1. *Minimal inhibitory concentration (in μg/ml) of the penicillins prepared from D- or L- enantiomorphs of α-phenoxypropionic, α-aminophenylacetic, or α-hydroxyphenylacetic acids*

| Organism | Penicillins prepared from: | | | | | |
|---|---|---|---|---|---|---|
| | α-Phenoxypropionic acid | | α-Aminophenylacetic acid | | α-Hydroxyphenylacetic acid | |
| | D | L | D | L | D | L |
| *Staphylococcus aureus*, Smith* | 0.12 | 0.03 | 0.06 | 0.25 | 0.05 | 0.2 |
| *Staphylococcus aureus*, 52-75† | 6.2 | 3.1 | 25 | 12.5 | 25 | 12.5 |
| *Diplococcus pneumoniae* | 0.06 | 0.06 | 0.1 | 0.8 | 0.1 | 6.2 |
| *Streptococcus pyogenes*, Digonnet | 0.15 | 0.03 | 0.1 | 0.4 | 0.05 | 0.1 |
| *Escherichia coli* | >100 | >100 | 6.2 | 25 | 12.5 | 50 |
| *Klebsiella pneumoniae* | >100 | >100 | 1.6 | 12.5 | 6.2 | 12.5 |
| *Salmonella typhosa* | >100 | >100 | 3.1 | 12.5 | 3.1 | 25 |

*Penicillin G sensitive.
†Penicillin G resistant.

Thus, there appears to be an anomaly in regard to the relation of biological activity to the configuration of the side chain in the different series. However, as will be shown below, this is only an apparent anomaly due to the convention used in designating asymmetric compounds D or L. Actually, the most active diastereoisomers have similar spatial relationships in the three series examined.

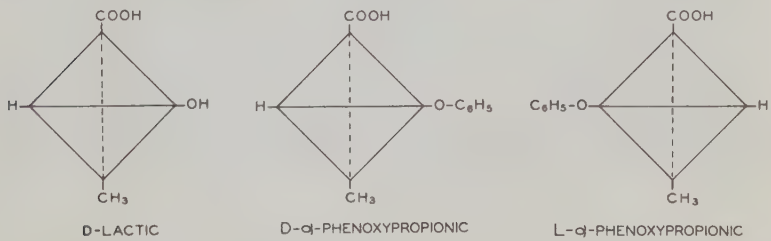


FIG. 1. *Fischer-Rosanoff projections of D-lactic and D- and L-α-phenoxypropionic acids*

In Fig. 1 are presented the Fischer-Rosanoff projection of D-lactic acid together with similar projections of D- and L-α-phenoxypropionic acids. The phenoxypropionic acids are considered to be phenol ethers of lactic acid in these projections. The D-α-phenoxypropionic acid, is thus, the ether of D-lactic acid.

In Fig. 2 the same type of comparison is made with D-lactic, D-α-hydroxyphenylacetic, and D-α-aminophenylacetic acids. In this case the configuration of the α-hydroxyphenylacetic acid is derived from that of D-lactic acid by replacement of the methyl group of the lactic acid with the phenyl group in hydroxyphenylacetic acid. The configuration of the D-α-aminophenylacetic acid is derived from that of the α-hydroxyphenylacetic acid, by substitution of the amino group for the hydroxyl group.

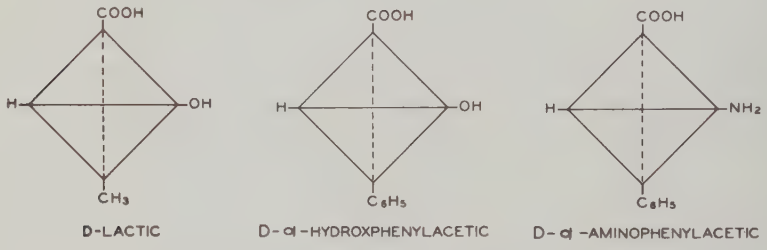


FIG. 2. *Fischer-Rosanoff projections of D-lactic, D-α-hydroxyphenylacetic, and D-α-aminophenylacetic acids.*

If α-phenoxypropionic acid is reoriented to facilitate comparison, the results depicted in Fig. 3 are obtained. The tetrahedron representing the asymmetric carbon is turned in such a way that the side connecting COOH to $O\text{-}C_6H_5$ groups is below the plane of the picture. The remaining two groups will be above the plane of the picture. In

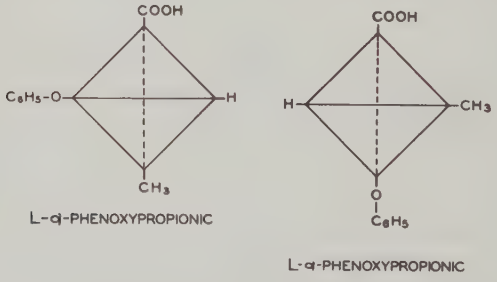


FIG. 3. *Two different projections of L-α-phenoxypropionic acid.*

this situation the $CH_3$ group will be to the right and the H atom will be to the left.

The reoriented projection of L-α-phenoxypropionic acid is compared with the proejctions of D-α-amino and D-α-hydroxyphenylacetic acids in Fig. 4. Viewing the representation of the three compounds this way, the similarity in structure becomes immediately apparent. Oriented as in this figure, the carboxyl group at the top and the bulky group (phenyl or phenoxy) at the bottom are below the plane, as indicated by the dotted line. The hydrogen atom will then fall to the left and the remaining substituent (methyl, amino, or hydroxy) to the right.

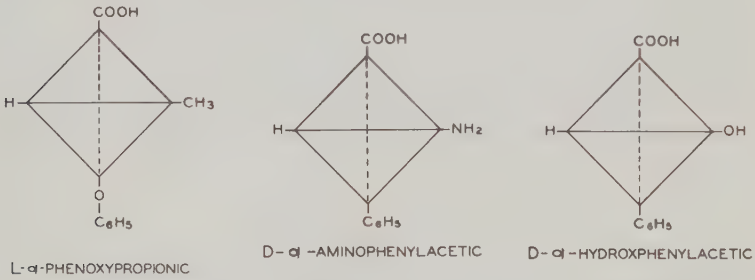


FIG. 4. *Projections of the three acids, precursors of the more active penicillins.*

Figure 5 is a photograph of a Courtauld (Hartley and Rolinson, 1952) model of the three most active compounds. It will be seen

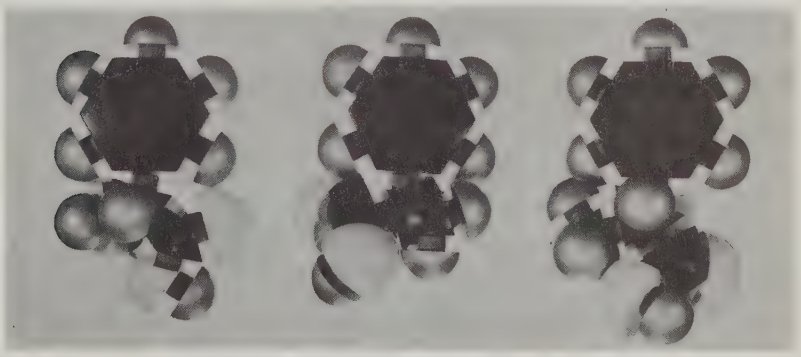


FIG. 5. *Comparisons of the Courtauld models of the three acids, precursors of the more active penicillins.*

that the structural relationship of the L-α-phenoxypropionic acid is similar to that of the two D isomers of α-amino and α-hydroxyphenylacetic acids, if one orients the molecule with respect to the bulky group.

## Discussion

Previous analyses of the properties of different penicillins have shown that the side chain can greatly modify factors such as activity, acid stability, and resistance to penicillinase. The fact that, in three different series of penicillins examined, the most active diastereoisomers have similar spatial relationships in the side chain indicates the importance of steric relationships in regard to activity. This is not surprising since, in all probabilities, the penicillin molecule must bind to certain sites as the primary event. Presumably, there is a preferred spatial configuration involving the side chain that is better satisfied by the more active diastereoisomers.

## Literature Cited

Batchelor, F. R., F. P. Doyle, J. H. C. Naylor, and G. N. Rolinson. 1959. Synthesis of penicillin: 6-aminopenicillanic acid in penicillin fermentations. Nature **183**:257.

Claridge, C. A., A. Gourevitch, and J. Lein. 1960. Bacterial penicillin amidase. Nature **187**: 237-238.

Hartley, G. S., and C. Rolinson. 1952. Atomic models. I. A new type of space filling atomic models. Trans. Faraday Soc. **48**:847-855.

Hudson, C. S. 1948. Historical aspects of Emil Fischer's fundamental conventions for writing stereo-formulas in a plane. Advances in Carbohydrate Chem. **3**:1-22.

Kaufman, W., and K. Bauer. 1960. Enzymatische Spaltung und Resynthese von Penicillin. Naturwissenschaften **47**:474-475.

Perron, Y. G., L. B. Crast, W. J. Gottstein, W. F. Minor, and L. C. Cheney. 1960*a*. Synthesis of potassium penicillin-152 potassium (-phenoxyethyl)-penicillin and both diastereoisomers. Antibiotics Ann. 1959-1960, p. 107-110.

Perron, Y. G., W. F. Minor, C. T. Holdrege, W. J. Gottstein, J. C. Godfrey, L. B. Crast, R. B. Babel, and L. C. Cheney. 1960*b*. Derivatives of 6-aminopenicillanic acid. I. Partially synthetic penicillins prepared from -aryloxyalkanoic acids. J. Am. Chem. Soc. **82**:3934-3938.

Rolinson, G. N., F. R. Batchelor, D. Butterworth, J. Cameron-Wood, M. Cole, G. C. Eustace, M. V. Hart, M. Richards, and E. B. Chain. 1960. Formation of 6-aminopenicillanic acid from penicillin by enzymatic hydrolysis. Nature **187**:236-237.

# PREPARATION AND PROPERTIES OF 3,4-DICHLORO-α-METHOXYBENZYLPENICILLIN

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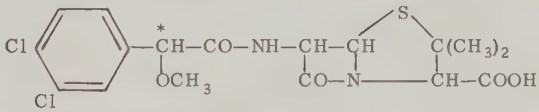
Examination of several α-substituted benzylpenicillins has revealed that the 3,4-dihalogeno-α-methoxybenzyl derivatives, and in particular 3,4-dichloro-α-methoxybenzylpenicillin (no. 356),



gave high and prolonged concentrations in plasma and organs of mice (Claesen et al., *in press*). We found similar prolonged blood levels in other animals and in humans (Van Dijck et al., *in press*).

As penicillin 356 contains an additional asymmetric carbon atom (marked by an asterisk in the formula), the product may contain different amounts of the two epimers. We have prepared the two diastereoisomers in the pure state and have examined their antibacterial activity.

Absorption and excretion of this penicillin in humans were also studied under various conditions.

## Materials and Methods

*Penicillins.* Penicillins G, V, phenethicillin, 3-4-dichlorophenethicillin, and 356 (Rixapen, R.I.T., s.a. Genval, Belgium) were used in the form of sodium or potassium salts.

Samples of 3,4-dichloro-α-methoxybenzyl penicillin sodium salt (no. 356) with $[\alpha]_D$ 210 to 220 (c = 1% in water) were used unless stated otherwise.

Pure epimers of this penicillin were prepared by fractional crystallization. When acetone (400 ml) was added to a solution of 25 g of penicillin 356 in 20 ml of water, a first crop (6.45 g) was obtained with $[\alpha]_D^{20}$ + 182 (c = 1% in water), mp 232 to 235 C (dec), purity: 97% (iodometric assay).

Analysis of $C_{17}H_{17}Cl_2N_2O_5SNa$. Calculated: C, 44.85; H, 3.76; N, 6.15; Cl, 15.57. Found: C, 44.82; H, 3.70; N, 5.89; Cl, 15.30.

The mother liquors were evaporated to a small volume, and water was removed by azeotropic distillation with butyl acetate at low temperature. After trituration with acetone, a second crop with $[\alpha]_D^{20}$ + 207 was obtained. Addition of ether to the mother liquors gave a third crop with $[\alpha]_D^{20}$ + 261 (c = 1% in water); mp 180 to 183 C (dec); purity: 95% (iodometric assay). Analysis. Found: C, 44.58; H, 4.16; N, 5.73; Cl, 14.95.

D-3,4-Dichloro-α-methoxybenzylpenicillin ($[\alpha]_D$ + 177) was also prepared by the condensation of 6-aminopenicillanic acid with D-3,4-dichloro-α-methoxyphenylacetyl chloride. The other epimer ($[\alpha]_D$ + 257) was obtained with the enantiomeric acid chloride; D or L-3,4-dichloro-α-methoxyphenylacetyl chloride was prepared by reaction of thionylchloride with D (-) or L(+)-3,4-dichloro-α-methoxyphenylacetic acid, mp 74 C and $[\alpha]_D \pm 98$ (c = 1% in ethanol ).

*In vitro activity.* The minimal inhibitory concentrations were determined by the serial tube dilution technique, using soy-Trypticase broth (Baltimore Biological Laboratory). A 1% dilution of a 20-hr culture was used as inoculum. The change of color of the phenol red indicator or the presence or absence of growth was determined after 18 hr incubation at 37 C.

*Blood and urine concentrations.* The activity in blood plasma was determined by the twofold dilution technique in soy-Trypticase broth with phenol red as the indicator and *Streptococcus pyogenes* ATCC 8668 as the test organism. The urine was assayed by the cylinder plate method, using *Staphylococcus aureus* 6538P.

Identical doses of the penicillins to be compared were administered on the same day to different animals or humans, and all blood and urine samples were assayed simultaneously against one or two microorganisms.

## Results

*Antibacterial spectrum.* The activity of penicillin G, V, phenethicillin, of the racemic mixture, and of the D and L isomers of penicillin 356, against sensitive microorganisms is given in Table 1. These results show that the bacteriostatic potency of the racemic mixture is generally identical with that of the most active isomer and is comparable with that of the other penicillins.

*Blood levels in rabbits.* A dose of 10 mg/kg of penicillin G, phenethicillin, 3,4-dichlorophenethicillin, or 356 (sodium salt) was injected intramuscularly into each test rabbit. Blood samples were taken by heart puncture 1, 3, and 6 hr after the injection. The antistreptococcal activity of the plasma (average of ten animals) is shown in Fig. 1. The rate of disappearance from the blood of penicillin G

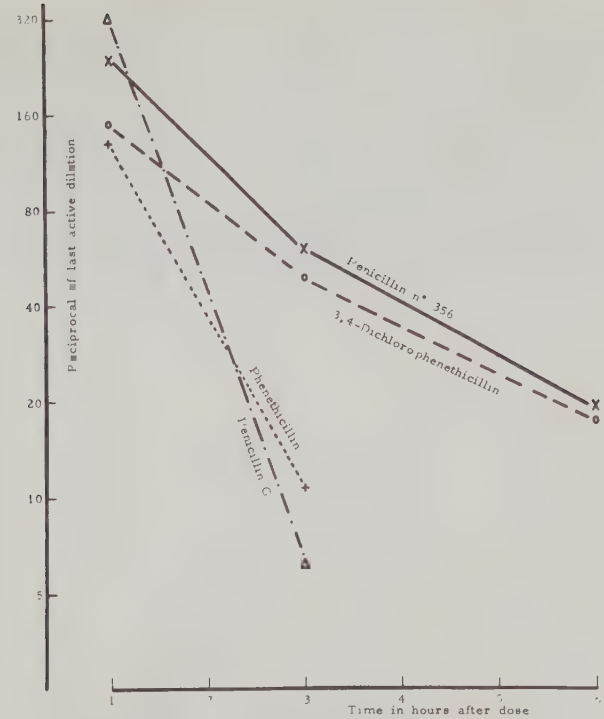



FIG. 1. *Average antistreptococcal activity of plasma (expressed as geometric means) after intramuscular injection of 10 mg/kg of penicillin G, phenethicillin, 3,4-dichlorophenethicillin, and penicillin 356 into rabbits.*

TABLE 1. *Minimal inhibitory concentrations of different penicillins*

| Test organism | Minimal inhibitory concentrations (μg/ml) | | | | | |
|---|---|---|---|---|---|---|
| | Penicillin 356 | | | Phene-thicillin | Penicillin G | Penicillin V |
| | Racemic mixture | D isomer | L isomer | | | |
| *Staphylococcus aureus* ATCC 6538P | 0.037 | 0.037 | 0.05 | 0.05 | 0.03 | 0.02 |
| *Streptococcus pyogenes* ATCC 8668 | 0.012 | 0.012 | 0.015 | 0.025 | 0.012 | 0.012 |
| *Diplococcus pneumoniae* NCTC 7465 | 0.015 | 0.007 | 0.06 | 0.015 | 0.015 | 0.015 |
| *Neisseria catarrhalis* NCTC 3632 | 0.037 | 0.025 | 0.06 | 0.06 | 0.03 | 0.05 |
| *Corynebacterium pseudodiphtheriticum* | 0.2 | 0.2 | >0.2 | 0.25 | 0.15 | 0.15 |

and phenethicillin is similar, but the clearance of penicillin 356 and 3,4-dichlorophenethicillin is much slower. The difference of activity is most marked 3 hr after the injection.

*Absorption and excretion in humans.* Phenethicillin (potassium salt) and penicillins V and 356 (sodium salts) were weighed in gelatin capsules. The amount used corresponded to 250 mg of acid. These capsules were given to healthy adult human volunteers who had fasted overnight. Breakfast was taken 1 hr later. Blood was withdrawn 1, 3, and 4 or 5 hr after the absorption of penicillin. The average comparative activity of the plasma is shown in Fig. 2.

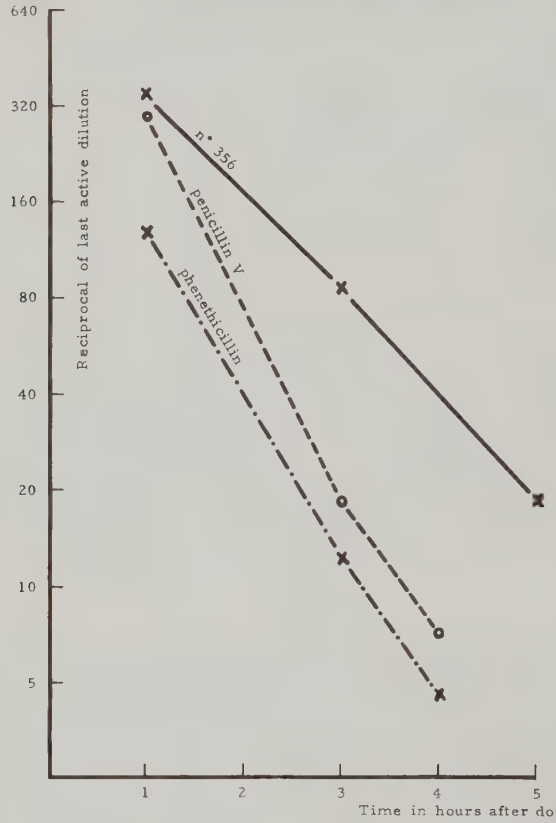



FIG. 2. *Average antistreptococcal activity of plasma (expressed as geometric means) after a single oral dose (before breakfast) of 250 mg (as acid) sodium penicillin V (eight subjects), potassium phenethicillin (eight subjects), and sodium penicillin 356 (17 subjects).*

The influence of food on the absorption and excretion of penicillin 356 was examined in another experiment. The same amount

(250 mg) of this penicillin was administered to humans before and just after breakfast. The peak levels obtained in nonfasting individuals were lower (Fig. 3). The maximum was reached 3 hr after a postbreakfast dose, compared with 30 min to 1 hr in fasting persons. The blood levels, however, were much more prolonged after the postbreakfast dosage. An antistreptococcal titer of 1:37 was obtained after 7 hr, which means that a dilution of 1:5 would not occur until after 10 hr. The same proportion of the dose was recovered in the urine: 38% in the fasting and 35% in the nonfasting state, indicating that the same amount of penicillin was absorbed under both conditions.

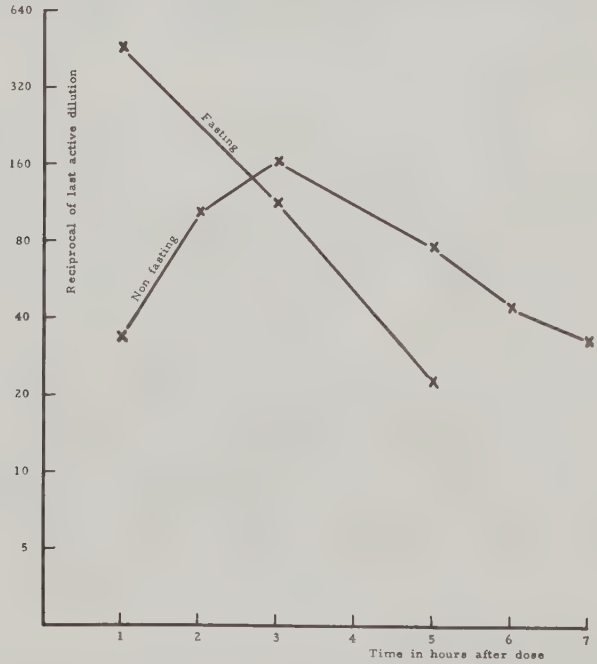



FIG. 3. *Average antistreptococcal activity of plasma (expressed as geometric means) after a single oral dose of 250 mg of penicillin 356, before and after breakfast (7 and 12 subjects).*

## Discussion

Previous work has shown that the antibacterial spectra of 3,4-dichloro-α-methoxybenzylpenicillin (no. 356), penicillin G, and penicillin V are similar, but that higher and more sustained blood and organ concentrations are obtained with penicillin 356 (Claesen et al.,

*in press*). The therapeutic significance of this observation was confirmed by mice-protection tests. The dose necessary to protect mice against an experimental infection induced by *Streptococcus pyogenes* or by *Staphylococcus aureus* was lower for penicillin 356 than for penicillin V (Van Dijck et al., *in press*).

We have now demonstrated that the antibacterial activity of the racemic mixture of penicillin 356 is practically identical with that of the most active epimer and that it can be used in vivo.

The oral resorption of different penicillins was compared in humans by determination of the maximal number of times the plasma could be diluted and still inhibit the growth of a pathogen. Jones and Finland (1957) and Perry, Hall, and Kirby (1959) demonstrated the importance of this method for evaluating different antibiotics. This method also has been adopted by other authors (Griffith, 1960; Martin and Nichols, 1960).

The antistreptococcal activity of blood obtained after oral administration of 250 mg of penicillin V or phenethicillin is of the same order of magnitude as that observed in other laboratories (McCarthy and Finland, 1960; Griffith, 1960). Penicillin 356 gives the same antibacterial titer after 1 hr. Previous studies have shown that penicillin V and phenethicillin yield a maximum after 30 min, whereas penicillin 356 gives the same plasma levels after 30 and 60 min (Van Dijck et al., *in press*).

The most significant distinction between these penicillins is in their excretion. Whereas an antibacterial blood level of 1:5 to 1:8 is observed 4 hr after administration of penicillin V and phenethicillin, the same level is not reached with penicillin until after 7 hr. The prolonged blood levels are still more marked when penicillin 356 is taken with food. A therapeutic level is present throughout a 10-hr period. As the same amount of antibiotic is excreted in the urine by individuals who have taken this penicillin before and after breakfast, we may assume that the same amount has been absorbed. The curve of antistreptococcal activity after a postbreakfast dose is similar to, but generally higher than, that observed after administration of 250 mg of propionyl erythromycin lauryl sulfate (Stephens, Conine, and Murphy, 1959).

We have some indications of the factors that cause these more prolonged blood levels. A delayed absorption must certainly be considered when penicillin 356 is taken with food. However, more prolonged blood levels were also observed after injection of penicillin 356 in rabbits and in humans. We also observed prolonged blood levels after intramuscular injection of 3,4-dichlorophenethicillin into rabbits. These results show that the metabolism of penicillin was favorably influenced by the introduction of chloro substituents in the 3,4-position of the benzene ring. As the in vitro activity of 3,4-dichlorophenethicillin was not better, and with some strains was lower, than that of penicillin 356, this product was not further examined.

Although some difference in removal by the kidneys is not excluded, it is more likely that excretion is retarded by the lipid solubility. The influence of lipid solubility on the metabolism of drugs was reviewed recently by Brodie and Hogben (1957). Penicillin 356 is more soluble in several organic solvents than are penicillins V and G. The storage in different tissues may also explain some other differences in metabolism. Whereas some 10 to 15% of the *p*-hydroxy-derivative of penicillin V is present in the urine, the inhibition zone of the more polar metabolite of penicillin 356, observed after bioautography of the paper chromatogram, is practically as large as that of the unchanged product (Parmentier, *unpublished data).* Prolonged excretion in the bile and reabsorption in the intestine can explain this greater amount of metabolite.

## Literature Cited

Brodie, B., and C. A. Hogben. 1957. Some physicochemical factors in drug action. J. Pharm. and Pharmacol. **9**:345-380.

Griffith, R. S. 1960. Comparison of antibiotic activity in sera after the administration of three different penicillins. Antibiotic Med. & Clin. Therapy **7**:129-135.

Jones, W. F., and M. Finland. 1957. Tetracycline, erythromycin, oleandomycin and spiramycin, and combinations of tetracycline with each of the three other agents – comparisons of activity in vitro and antibacterial action of blood after oral administration. New Engl. J. Med. **257**:481-491.

McCarthy, C. G., and M. Finland. 1960. Absorption and excretion of four penicillins. New Engl. J. Med. **263**:315-326.

Martin, W. J., and D. R. Nichols. 1960. Penicillin V and phenethicillin potassium in serum: comparison of concentrations and of antibacterial effects. Proc. Staff Meetings Mayo Clinic **35**: 577-584.

Perry, D. M., G. A. Hall, and W. M. Kirby. 1959. Triacetyloleandomycin and erythromycin: a comparison of in vitro activity and antibacterial action of blood after oral administration. Antibiotic Med. & Clin. Therapy **6**:347-352.

Stephens, V. C., J. W. Conine, and H. W. Murphy. 1959. Esters of erythromycin. IV. Alkyl sulfate salts. J. Am. Pharm. Assoc., Sci. Ed. **48**:620-622.

# EVALUATION OF THE BIOLOGICAL ACTIVITIES OF ALPHA-PHENOXYPROPYL PENICILLIN

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Alpha-phenoxypropyl penicillin has two epimeric forms. The one prepared by condensing *l*-α-phenoxy-*n*-butyric acid with penicillin nucleus has considerably more biological activity than the other prepared from *d*-α-phenoxy-*n*-butyric acid. The *l*-α-phenoxypropyl penicillin [6-(*l*-α-phenoxy-*n*-butyramido)penicillanic acid] has been given the generic name levopropylcillin. Data for in vitro and in vivo activities of eight penicillins, *l*-α-phenoxypropyl, *d*-α-phenoxypropyl, *dl*-α-phenoxypropyl, phenoxymethyl, *dl*-α-phenoxyethyl, *l*-α-phenoxyethyl, *d*-α-phenoxyethyl, and dimethoxyphenyl have been collected.

## Materials and Methods

The in vitro sensitivities of the test organisms (Table 1) to the various penicillins were determined by conventional serial tube-dilution procedures. The sensitivities of *Diplococcus pneumoniae* strain Park I and *Streptococcus pyogenes* strain C-203 were also assessed by a more accurate disc-plate method.

The organisms employed in the experimental mouse infections were selected because of their virulence for mice. Groups of 20 Swiss mice per dose level of penicillin were used in these tests. In every case, a sufficient number of organisms was injected intraperitoneally to cause death of untreated control animals within 7 days. The bacterial suspensions were prepared from 18-hr broth cultures and diluted in saline, or beef-infusion neopeptone broth, to a suitable challenge dose. Final infecting dilutions for *Staphylococcus aureus* were prepared in 5% gastric hog mucin. Twofold dilutions of the penicillins were employed to treat the mice 1 and 5 hr after infection. The mice were observed for a period of 7 days and the $LD_{50}$ and $ED_{50}$ values calculated according to the method of Reed and Muench (1938).

Tests with *Borrelia novyi* were conducted essentially as described by McCowen et al. (1953). A single dose of the antibiotic

TABLE 1. *Minimal inhibitory concentrations of penicillins required for bacteria by conventional tube-dilution procedures**

| Organism | *l*-α-Phenoxypropyl penicillin | *d*-α-Phenoxypropyl penicillin | *dl*-α-Phenoxypropyl penicillin (50/50 *dl*) | Phenoxymethyl penicillin | *dl*-α-Phenoxyethyl penicillin | *l*-α-Phenoxyethyl penicillin | *d*-α-Phenoxyethyl penicillin | Dimethoxyphenyl penicillin |
|---|---|---|---|---|---|---|---|---|
| *D. pneumoniae* Park I | 0.0312 | 0.0625 | 0.0312 | 0.0625 | 0.25 | 0.0625 | 0.0625 | 0.125 |
| *D. pneumoniae* Parker II | 0.0312 | 0.0625 | 0.0625 | 0.0312 | 0.25 | 0.0625 | 0.125 | 0.25 |
| *S. pyogenes* C 203 | 0.125 | 0.125 | 0.0312 | 0.0625 | 0.0625 | 0.0156 | 0.0312 | 0.25 |
| *C. diphtheriae* #42 | 0.5 | 0.25 | NT† | 0.0625 | 0.25 | NT | NT | >1.0 |
| *C. perfringens* #BP6K | 1.0 | 0.5 | NT | 0.25 | 0.5 | NT | NT | 1.0 |
| *C. tetani* Harvard | 0.25 | 0.25 | NT | 0.5 | 1.0 | NT | NT | >1.0 |
| *N. gonorrheae* | 0.0312 | 0.0156 | NT | 0.0156 | 0.125 | NT | NT | 0.25 |
| *N. meningitidis* ATCC 6253 | < 0.0039 | 0.0625 | NT | 0.25 | 0.25 | NT | NT | 0.5 |
| *S. aureus* 3055 | 0.0312 | 0.125 | 0.0312 | 0.0312 | 0.125 | 0.0156 | 0.0312 | 1.95 |
| *S. aureus* 3074 | 0.98 | 0.98 | 0.98 | 15.6 | 0.78 | 0.78 | 1.56 | 1.95 |
| *S. aureus* H43 | 7.8 | 62.5 | 5.0 | 125 | 12.5 | 6.25 | 12.5 | 3.9 |
| *S. aureus* H114 | 1.95 | 31.25 | 2.5 | 62.5 | 12.5 | 12.5 | 12.5 | 3.9 |
| *S. aureus* H225 | 1.95 | 7.8 | 2.5 | 125 | 12.5 | 12.5 | 12.5 | 1.95 |
| *S. aureus* H232 | 7.8 | 7.8 | 5.0 | 125 | 12.5 | 12.5 | 25.0 | 1.95 |

*All figures expressed as μg/ml. Inoculum was approximately 100,000 bacteria/ml. Trypticase soy broth was used for aerobic bacteria and thioglycolate medium for anaerobes with 5% blood added to broths used for *D. pneumoniae*, *S. pyogenes*, and *Neisseria*. MIC was determined visually after 24 hr of incubation at 37 C.

†Not tested.

was given approximately 18 hr after the mice were infected intraperitoneally with *B. novyi*. At 1, 2, 3, 5, and 24 hr after treatment, tail blood from each mouse was examined by a dark-field technique for the presence of spirochetes.

An in vivo method was devised for quantitatively estimating the therapeutic activity of penicillin in the presence of penicillinase. The procedure utilizes *S. pyogenes* as the indicator organism. After intraperitoneal infection (0.5 hr) with *S. pyogenes*, 500 units of penicillinase derived from *Bacillus cereus* or *S. aureus* were injected subcutaneously into groups of 10 mice. Twofold dilutions of penicillin, given in a single subcutaneous dose, were administered 1 hr after infection to the penicillinase-treated mice and to mice receiving no penicillinase. The $ED_{50}$ values were calculated after 7 days. The relative resistance of the various penicillins to inactivation by penicillinase in vivo can be noted by comparing the differences in the amounts required for therapeutic effects in the presence and absence of the enzyme.

## Results and Discussion

The in vitro sensitivity data obtained by the tube-dilution procedure (Table 1) indicate *l*-α-phenoxypropyl penicillin (levopropylcillin) is, in most cases, equivalent to, or more inhibitory than, the other seven penicillins. The *S. aureus* data suggest levopropylcillin has more inhibitory activity against penicillinase-producing strains; however, unlike dimethoxyphenyl penicillin, the minimal inhibitory concentrations (MIC) increase with larger inocula. This change in MIC, with an increase in inoculum size, is interpreted to mean levopropylcillin is not resistant to the action of penicillinase.

The results (Table 2) obtained in the pneumococcal infection show levopropylcillin to be two to four times more effective, by either route of administration, than any of the other penicillins tested. This superiority is not as evident in the data derived from the streptococcal test. In the latter, the effective dose of levopropylcillin was only about a quarter less than doses required for the other penicillins. The pneumococcus, because of the larger dose required, appears to be the better organism for determining therapeutic effectiveness of penicillins.

The in vitro sensitivity data for *D. pneumoniae* and *S. pyogenes* obtained by the more accurate disc-plate method indicate no differences large enough to account for greater therapeutic effectiveness of levopropylcillin (Table 3). Better absorption of this antibiotic appears the most likely explanation.

Levopropylcillin is not resistant to degradation by penicillinase; nevertheless, it is quite active in the test tube against moderate inocula of penicillinase-producing *S. aureus* strains (Table 1). In

TABLE 2. *$ED_{50}$ values derived from penicillin treatment of experimental Diplococcus pneumoniae and Streptococcus pyogenes infections in mice**

| Penicillin | *Diplococcus pneumoniae* | | *Streptococcus pyogenes* | |
|---|---|---|---|---|
| | Oral therapy | Subcutaneous therapy | Oral therapy | Subcutaneous therapy |
| *l*-α-Phenoxypropyl | 1.17 | 0.851 | 0.331 | 0.22 |
| *d*-α-Phenoxypropyl | 4.02 | 3.3 | 1.42 | 1.73 |
| *dl*-α-Phenoxypropyl | 1.45 | 1.58 | 0.453 | 0.34 |
| Phenoxymethyl | 2.08 | 3.49 | 0.431 | 0.183 |
| *dl*-α-Phenoxyethyl | 2.46 | 1.6 | 0.405 | 0.59 |
| *l*-α-Phenoxyethyl | 2.55 | 2.05 | 0.418 | 0.242 |
| *d*-α-Phenoxyethyl | 3.01 | 3.2 | 0.627 | 0.421 |
| Dimethoxyphenyl | 58.5 | 20.5 | 29.8 | 1.92 |
| Reed-Muench $LD_{50}$ challenge† | 347 | 302 | 4680 | 2920 |

*$ED_{50}$ values expressed as mg/kg × two treatments (1 and 5 hr postinfection).
†One $LD_{50}$ = challenge dose required to kill 50% of the mice.

TABLE 3. *Minimal inhibitory concentrations of penicillins required for Diplococcus pneumoniae and Streptococcus pyogenes determined by disc-plate method**

| Penicillin | *D. pneumoniae* Park I | *S. pyogenes* C 203 |
|---|---|---|
| | μg/ml | μg/ml |
| *l*-α-Phenoxypropyl | 0.1 | 0.06 |
| *d*-α-Phenoxypropyl | 0.2 | 0.06 |
| *dl*-α-Phenoxypropyl | 0.08 | 0.06 |
| Phenoxymethyl | 0.06 | 0.02 |
| *dl*-α-Phenoxyethyl | 0.2 | 0.06 |
| *l*-α-Phenoxyethyl | 0.2 | 0.06 |
| *d*-α-Phenoxyethyl | 0.2 | 0.06 |
| Dimethoxyphenyl | 0.4 | 0.4 |

*Blood agar seeded with $1.6 \times 10^5$ bacteria/ml. Dilutions of penicillin were dropped in 0.1–ml amounts to antibiotic assay discs, and the plates were incubated for 24 hr at 37 C. The lowest dilution producing a zone of inhibition was the minimal inhibitory concentration.

mice infected with a penicillinase-producing *S. aureus* strain, this penicillin appeared surprisingly active, the effective parenteral dose being only twice that of dimethoxyphenyl penicillin (Table 4). The data, resulting from the special tests for therapeutic activity in the presence of penicillinase, show no increased resistance in vivo for levopropylcillin to degradation by this enzyme (Table 5). However, the $ED_{50}$ values for levopropylcillin in the presence of either penicillinase were the lowest obtained for any of the penicillins tested.

Gourevitch et al. (1961) have demonstrated that, as the side chain of the α-phenoxyalkyl penicillins is lengthened, the inhibitory activity against penicillin-sensitive *S. aureus* decreases. This is

TABLE 4. *$ED_{50}$ values as derived for penicillin-sensitive and penicillinase-producing S. aureus infections in mice following penicillin treatment**

| Penicillin | Penicillin-sensitive *S. aureus* | | Penicillinase-producing *S. aureus* | |
|---|---|---|---|---|
| | Oral therapy | Subcutaneous therapy | Oral therapy | Subcutaneous therapy |
| *l*-α-Phenoxypropyl | 0.645 | 0.73 | 154 | 43.7 |
| *d*-α-Phenoxypropyl | 1.13 | 1.54 | 563 | 141 |
| *dl*-α-Phenoxypropyl | 0.403 | 0.92 | 94.6 | 78.6 |
| Phenoxymethyl | 0.313 | 0.108 | 475 | 278 |
| *dl*-α-Phenoxyethyl | 0.211 | 0.42 | 77.4 | 155 |
| *l*-α-Phenoxyethyl | 0.323 | 0.162 | 58.5 | 68.5 |
| *d*-α-Phenoxyethyl | 0.473 | 0.283 | 67.3 | 145 |
| Dimethoxyphenyl | 23.4 | 8.1 | 147 | 22.4 |
| Reed-Muench $LD_{50}$ challenge† | 583 | 1000 | 2180 | 59 |

*$ED_{50}$ values expressed as mg/kg × two treatments (1 and 5 hr postinfection).
†One $LD_{50}$ = challenge dose required to kill 50% of the mice.

TABLE 5. *Estimation of the therapeutic activity of penicillins in the presence of added penicillinase, using Streptococcus pyogenes as the infecting organism*

| Penicillin | *Bacillus cereus* penicillinase | | | *S. aureus* penicillinase | | |
|---|---|---|---|---|---|---|
| | $ED_{50}$* (no penicillinase) | $ED_{50}$ (with 500 units penicillinase)† | Fold difference | $ED_{50}$ (no penicillinase) | $ED_{50}$ (with 500 units penicillinase) | Fold difference |
| *l*-α-Phenoxypropyl | 0.34 | 5.0 | 13.7 | 0.61 | 2.82 | 4.64 |
| *d*-α-Phenoxypropyl | 3.94 | 43.5 | 12.1 | 3.65 | 13.3 | 3.65 |
| Phenoxymethyl | 0.672 | 22 | 32.8 | 0.95 | 8.65 | 9.1 |
| *dl*-α-Phenoxyethyl | 0.682 | 10.4 | 15.3 | 0.786 | 10.4 | 13.2 |
| Dimethoxyphenyl | 8.27 | 13.7 | 1.65 | 16.6 | 10.4 | 0.66 |
| Reed-Muench $LD_{50}$ challenge‡ | 1,000 | | | 3,915 | | |

*$ED_{50}$ values expressed as mg/kg. One subcutaneous dose of penicillin 1 hr after infection.
†One subcutaneous dose of penicillinase 0.5 hr after infection.
‡One $LD_{50}$ = challenge dose required to kill 50% of the mice.

TABLE 6. *Activity of penicillins on experimental Borrelia novyi infections in mice*

| Penicillin | Dose* | | Time in hr postinfection† | | | | |
|---|---|---|---|---|---|---|---|
| | Route | Mg/kg | 1 | 2 | 3 | 5 | 24 |
| *l*-α-Phenoxypropyl | oral | 5 | O | R | D | D | D |
| | ip | 5 | O | R | D | D | D |
| *d*-α-Phenoxypropyl | oral | 5 | O | R | R | D | D |
| | ip | 5 | O | O | R | R | D |
| *dl*-α-Phenoxypropyl | oral | 5 | O | R | D | D | D |
| | ip | 5 | O | R | D | D | D |
| Phenoxymethyl | oral | 50 | O | O | R | D | D |
| | oral | 5 | O | O | O | O | O |
| | ip | 50 | O | O | R | D | D |
| Infected controls | | | O | O | O | O | O |

*Dose = one treatment 18 hr after intraperitoneal (ip) inoculation with *B. novyi*.
†Tail blood examined for spirochetes by dark-field microscopy. O = No reduction in number of parasites; R = reduction in number of parasites; D = disappearance of parasites.

also true for levopropylcillin since the $ED_{50}$ values for sensitive *S. aureus* strain 3055 (Table 4) are greater than those obtained with phenoxymethyl or *dl*-α-phenoxyethyl penicillins.

The data obtained from *B. novyi* infection in mice (Table 6) show levopropylcillin to be 10 times more effective than phenoxymethyl penicillin either by the oral or intraperitoneal route. The spirochetes completely disappear from the blood stream within 3 hr with a single dose at a level of 5 mg/kg, whereas 50 mg/kg are required for phenoxymethyl penicillin to produce the same response in 5 hr.

## Acknowledgments

The authors express sincere appreciation to Lois Hawley, D. Holmes, and Barbara Simmons for invaluable laboratory assistance; to M. Callender for the *Borrelia novyi* testing; to H. Henry for preparation of *S. aureus* penicillinase; and to Vivian Hatcher, June Wood, Patricia Wood, and Mary Lassiter for their technical assistance.

## Literature Cited

Gourevitch, A., G. A. Hunt, J. R. Luttinger, C. C. Cermack, and J. Lein. 1961. Activity of α-phenoxyalkyl penicillins against sensitive and resistant staphylococci. Proc. Soc. Exptl. Biol. Med. **107**:455-458.

McCowen, M. C., M. E. Callender, J. F. Lawlis, Jr., and M. C. Brandt. 1953. The effects of erythromycin (Ilotycin, Lilly) against certain parasitic organisms. Am. J. Trop. Med. Hyg. **2**:212-218.

Reed, L. J., and H. Muench. 1938. A simple method for estimating 50 percent end points. Am. J. Hyg. **28**:493-497.

# STAPHYLOCOCCAL RESISTANCE TO METHICILLIN

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The only type of penicillin-resistant staphylococcus thought to be of clinical importance is that type which produces penicillinase. The nonpenicillinase penicillin-tolerant strains are mostly avirulent in nature and low in coagulase production. With the introduction of methicillin (2,6-dimethoxyphenyl penicillin), which is for the most part unaffected by penicillinase, the question arises as to whether the methicillin-tolerant strains, which may arise, will be avirulent as is the case with penicillin-tolerant strains. Elek and Fleming (1960) failed to produce methicillin resistance in 12 staphylococcal strains after 20 serial passages on gradient plates. Thompson, Harding, and Simon (1960) and Stewart, Nixon, and Coles (1960) also failed to produce resistance. Thus Elek and Fleming expect that if methicillin-resistant mutants do develop, they will be few and of little virulence. Since these papers have appeared, three naturally occurring strains have been reported with resistance up to 25 μg/ml (Barber, 1961). Also, Steinman (1961) in this country and Barber (1961) and Knox and Smith (1961) in England have produced laboratory resistant strains.

This paper deals with some of the characteristics of methicillin-resistant staphylococci trained in the laboratory.

## Materials and Methods

Resistance was developed in methicillin broth by passing the organisms in increasing concentrations of the antibiotic. Large numbers of organisms were used in the inoculum by scooping them from solid media. The organisms were grown on methicillin-free blood agar between passages. If an organism did not survive one of the passages it was discarded.

The degree of resistance was determined by both the minimal inhibitory concentration (MIC) and disc methods. Coagulase determinations were carried out on 18-hr broth cultures, with the time of plasma clotting noted. Virulence tests were performed by injecting

the organisms intravenously and intradermally into rabbits. Microbiological assay was used to test for methicillin destruction. The approximate number of organisms needed in the inoculum and the concentration of antibiotic where resistance may start were determined roughly by inoculating known numbers of organisms into different concentrations of the antibiotic.

## Results

Resistance up to 35 μg/ml was developed in 10 transfers. It was felt that this amount of resistance was ample for the study. All of the strains were sensitive to 3 μg/ml at the start, and the resistance had to be raised gradually, starting with a low drug concentration. Also, an inoculum of several billion organisms had to be used. Six out of 19 strains survived the procedure. A penicillinase-negative *Staphylococcus albus* strain, which grew very poorly after resistance had developed, was lost during storage. The other five strains were penicillinase-positive, coagulase-positive *Staphylococcus aureus;* two

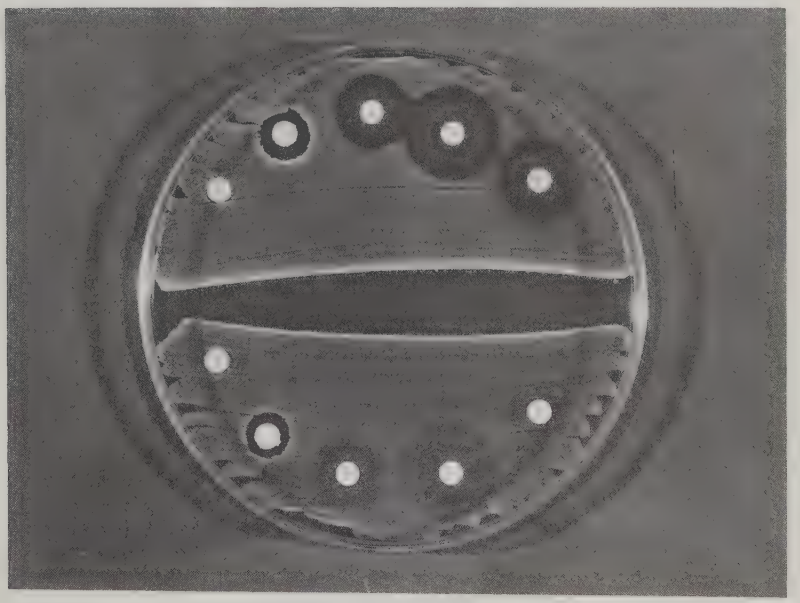

FIG. 1. *Collateral resistance to Prostaphlin. Parent strain is at the top and the methicillin-resistant mutant at the bottom. Discs from left to right are 2 units penicillin, 10 μg Syncillin, 2 and 10 μg Prostaphlin, and 5 μg methicillin.*

became resistant to 5 μg/ml of methicillin, one to 20 μg, and two to 35 μg/ml of methicillin. Collateral resistance to penicillin and Syncillin has been noted by others (Barber, 1961; Knox, 1961). Fig. 1 shows that collateral resistance also occurs with the new penicillin, Prostaphlin (5-methyl-3-phenyl-4-isoxazolyl penicillin). The MIC of Prostaphlin on the parent strain showed it to be sensitive to 0.5 μg and the methicillin-resistant strain to be resistant to 5 μg/ml. Although it appears that most strains which develop resistance to methicillin will show an increase in their resistance to penicillin G, Syncillin, and Prostaphlin, the reverse of this also can take place.

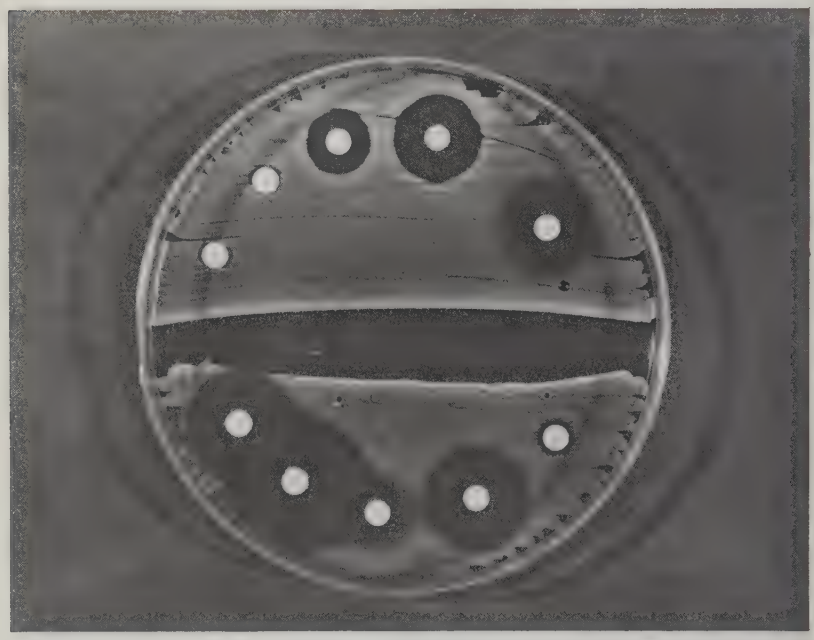

FIG. 2. *Loss of penicillinase of a staphylococcus on becoming methicillin-resistant. Parent strain is at the top and methicillin-resistant strain at the bottom of the plate. Discs are arranged as in Fig. 1.*

Figure 2 shows a strain in which the parent culture was resistant to penicillin and Syncillin. After resistance to methicillin was developed, it became sensitive to penicillin and Syncillin. The parent strain was a penicillinase producer, resistant to more than 110 units of penicillin G per ml, and the methicillin-resistant mutant became sensitive to 0.3 units of penicillin G. The resistance to methicillin appears stable. No reduction in resistance appeared in the five coagulase-positive strains after several months storage and subculturing

more than 12 times. Barber (1961), however, reports that penicillinase-negative strains tend to lose their resistance rapidly after subculturing on methicillin-free media.

There was no significant difference in the coagulase activity of the parent and resistant strains. The phage pattern also remained the same.

Methicillin is not destroyed by the resistant strains. Methicillin broth contained as much methicillin after resistant strains had grown in it for 18 hr as the control. The virulence of the two resistant strains tested was less than that of the parents. The four rabbits injected intravenously with the parent strain died 2 to 9 days after injection. One rabbit receiving a resistant strain died on the 10th day. Of the remaining three rabbits, two became ill with loss in weight. All three were sacrificed on the 23rd day. One had had a generalized infection with large abscesses covering the liver from which the staphylococcus was recovered. The other two appeared normal. The left side of the rabbit received five 0.2-ml intradermal injections of the 18-hr parent culture (Fig. 3). The right side was used for the injections of the resistant strain (Fig. 4). The five dilutions ranged from undilute to 1:16. The parent strain produced necrosis while its resistant strain produced only erythema.

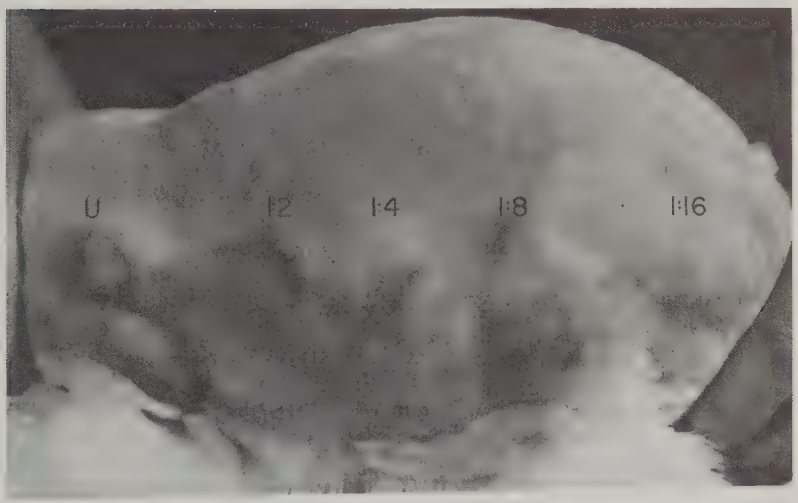


FIG. 3. *Effects of intradermal injections in a rabbit. Injections of 0.2-ml dilutions of parent strain into the left side of the rabbit. The five dilutions range from undilute to 1:16.*

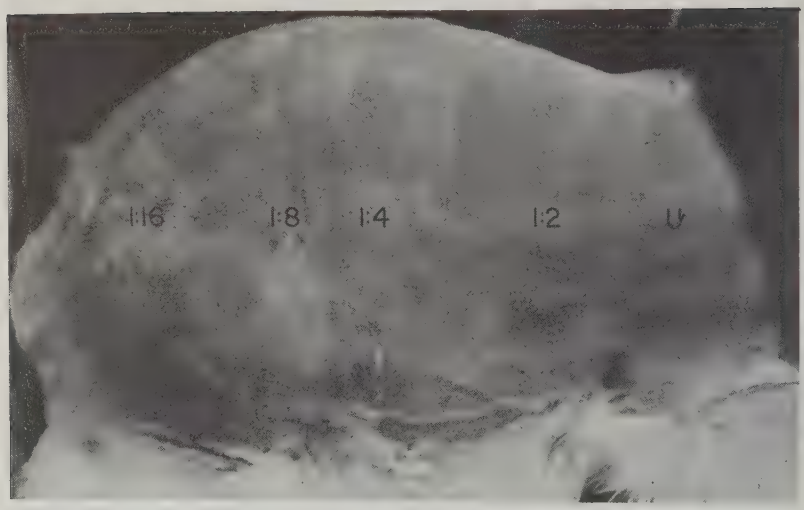


FIG. 4. *Effects of intradermal injections in a rabbit. Injections of 0.2-ml dilutions of methicillin-resistant mutant into the right side of the rabbit. The five dilutions range from undilute to 1:16.*

When the methicillin-resistant strain was recovered from the intravenously injected rabbit on the 23rd day, it was injected intradermally into another rabbit. The virulence appeared to have been increased to at least that of the sensitive strain without a loss in its resistance to methicillin.

A total of 18 billion staphylococci inoculated equally into 18 tubes of broth containing 10 and 20 $\mu$g methicillin per ml failed to produce a resistant mutant. However, one of the 18 tubes containing 5 $\mu$g/ml produced a mutant which proved (by the MIC method) to be resistant to 5 $\mu$g of the methicillin.

## Discussion

Staphylococci develop resistance to methicillin in a gradual manner, as they do when they become penicillin-tolerant. But it is too early to assume that they will also be less virulent and of as little importance as these penicillin-tolerant strains. The resistant organisms differ in that the penicillin-tolerant strains do not grow as well or produce as much coagulase as their parent strains. Also, their resistance is fairly unstable. Methicillin-tolerant strains, on the other hand, grow as well and produce as much coagulase as the parent strain. Their resistance also seems to be quite stable.

Although experiments here have shown the resistant strain to be somewhat less virulent, this may be due to its storage and transfers on synthetic media. Barber (1961) found that three penicillin-destroying methicillin-resistant strains were as virulent in mice as their parents. However, even though the resistant strains may be virulent, possibly they may never become much of a clinical problem. Out of several thousand strains tested thus far, only three naturally occurring resistant strains have been reported. Also, although the mutation rate has not been determined, it must be quite low. Out of 18 tubes containing methicillin and inoculated with a billion organisms each, only one tube produced a resistant mutant. Nevertheless, this does not eliminate the possibility of methicillin-resistant staphylococci from arising and spreading in the hospitals as in the cases with the other antibiotics.

## Literature Cited

Barber, M. 1961. Methicillin-resistant staphylococci. J. Clin. Pathol. 14:385-393.

Elek, S. D., and P. C. Fleming. 1960. New technique for the control of hospital cross-infection. Experience with BRL 1241 in a maternity unit. Lancet 2:569-572.

Knox, R., and J. J. Smith. 1961. The nature of penicillin resistance in staphylococci. Lancet 2:520-522.

Stewart, G. T., H. H. Nixon, and H. M. T. Coles. 1960. A report on clinical use of BRL 1241 in children with staphylococcal and streptococcal infections. Brit. Med. J. 5200:703-706.

Steinman, H. G. 1961. Biochemical studies on 6-aminopenicillanic acid, benzyl penicillin, and 2,6-dimethoxyphenyl penicillin, p. 17-25. *In* P. A. Bunn, [ed.], A symposium on the new dimethoxyphenyl penicillin. Syracuse University Press, Syracuse.

Thompson, R. E. M., J. W. Harding, and R. D. Simon. 1960. Sensitivity of *Staphylococcus pyogenes* to benzyl penicillin and BRL 1241. Brit. Med. J. 5200:708-709.

# OBSERVATIONS CONCERNING PROTEIN INTERFERENCE AND PHARMACODYNAMIC BEHAVIOR OF NINE PENICILLINS

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Isolation of 6-aminopenicillanic acid, the nucleus of the penicillin molecule, by Batchelor and coworkers (1959) has been recognized as an important discovery. It is now possible by modification of the side chain to prepare a wide variety of semisynthetic penicillins having differing biological and pharmacological characteristics. Two such agents, phenethicillin and methicillin, have been commercially available for some time. Numerous reports (McCarthy, Wallmark, and Finland, 1961; Cronk et al., 1960; Knudson and Rolinson, 1959; Griffith, 1960; McCarthy and Finland, 1960; McCarthy, Hirsh, and Finland, 1960) concerning the antimicrobial spectrum, absorption, fate, excretion, and serum antimicrobial activity of these and certain other semisynthetic penicillins have appeared in the literature.

These data, however, for the most part, have not considered the effect of the presence of human serum or other protein in the test system. It is well known that antimicrobial compounds may lose all or part of their activity in the presence of serum. Davis (1943) and Tompsett, Shultz, and McDermott (1947) demonstrated serum protein binding of certain sulfonamides and penicillins X, G, dihydro F, and K. Tompsett and coworkers demonstrated that in vitro this phenomenon was associated with a loss of antibacterial efficiency, the extent of which was directly related to the degree of protein binding. In one instance, penicillin K, the presence of 30% human serum caused a profound (93%) loss of antibacterial effect. This striking serum interference appeared to be the reason for the discrepancy between the in vitro and in vivo activity of penicillin K in experimental animal infections observed by Coghill, Osterberg, and Hazel (1946), Hobby, Burkhart, and Hyman (1946), and Eagle and Musselman (1946). It was further observed (Committee on Medical Research, U.S. Public Health Service and Food and Drug Administration, 1946) that mixtures of penicillins that contained increased amounts of penicillin K exhibited decreased effectiveness when employed in the treatment of human syphilis. More recently, Kunin (1961) showed by dialysis experiments that two other penicillins, phenethicillin and

phenylmercaptomethyl penicillin, are bound to serum protein, and Gourevitch et al. (1961) demonstrated decreased antimicrobial activity of several alpha-phenoxyalkyl penicillins in the presence of 50% human serum.

The purpose of this report is to present certain observations concerning the effect of bovine albumin and pooled human serum on the antimicrobial activity of nine penicillins, and the results of pharmacological studies on five of the nine agents.

## Materials and Methods

The following penicillins were used in this study: benzyl (G), which was supplied by E. R. Squibb & Sons, New Brunswick, N.J.; phenylmercaptomethyl (PMM) and alpha-phenoxypropyl (POP), supplied by Eli Lilly & Co., Indianapolis, Ind.; phenoxymethyl (V), alpha-phenoxyethyl (POE), and 2-ethoxy-1-naphthyl (EON), obtained from Wyeth Laboratories Inc., Radnor, Pa.; alpha-phenoxyisobutyl (POIB), supplied by Chas. Pfizer & Co., Inc. Groton, Conn.; and 2,6-dimethoxyphenyl (DMP) and 5-methyl-3-phenyl-4-isoxozolyl (MPI), which were obtained from Bristol Laboratories, Syracuse, N.Y. Sterile standard reference powders of each agent were employed for susceptibility tests, and commercial lot preparations were utilized in the pharmacological crossover studies.

Susceptibility tests were performed by the standard serial two-fold dilution method (Grove and Randall, 1955). The diluents employed were Trypticase soy broth, bovine albumin in Trypticase soy broth (1.75 and 7%), pooled human serum in Trypticase soy broth (50%), and whole pooled human serum. Control studies indicated that, with the exception of whole serum the various diluents neither inhibited nor enhanced growth of the test organisms. Whole serum inhibited growth of certain strains of test organisms, and it was noted that this inhibitory effect varied from pool to pool of serum. The test organisms employed in the whole-serum experiments were those known not to be inhibited by this concentration of serum. Inoculum was 0.1 ml of a $10^2$ dilution of an 18-hr broth culture of the test organism in the appropriate diluent. This inoculum contained $10^5$ to $10^7$ viable cells. The minimal inhibitory concentration (MIC) was taken to be the concentration of antibiotic in the last tube of the series in which there was no visible growth.

The MIC of the various antibiotics for 21 strains of Viridans streptococci, 24 strains of nonpenicillinase-producing staphylococci, 76 strains of penicillinase-producing staphylococci, and 9 strains of enterococci was determined in the Trypticase soy broth diluent. All test organisms were clinical isolates. All Viridans streptococcal and enterococcal strains and some of the staphylococcal strains had

been isolated from patients with bacterial endocarditis. Details of these studies will be published elsewhere.

In brief summary, G, V, and POP exhibited similar antimicrobial activity against the Viridans streptococci and the nonpenicillinase-producing staphylococci. In this system, these agents were on an average 25 times more active than DMP and three times more active than MPI and EON. The penicillinase-producing strains of staphylococci were uniformly sensitive to DMP, MPI, and EON. MPI and EON were similar in activity and were on the average eight times more active than DMP. A very wide range of activity was observed with POP. The enterococcal strains were most sensitive to penicillin G and least sensitive to MPI.

A representative strain of Viridans streptococcus (I.D. 122) and a nonpenicillinase-producing staphylococcus (I.D. White) were employed as indicator organisms to study the effect of protein in the test system. Twofold dilution tests were carried out in parallel utilizing Trypticase soy broth and the various protein solutions as diluents. The efficiency of each agent was calculated using the formula:

$$\frac{\text{MIC in broth}}{\text{MIC in serum}} \times 100 = \text{percentage of efficiency}$$

Single dose crossover studies in groups of ten fasting healthy adults were carried out on two occasions. In the first study the subjects received intramuscular DMP, and MPI and penicillin V which were administered by the oral route. Orally administered penicillin V, POP, and intramuscular, crystalline penicillin G were compared in the second study. Venous blood was drawn at 0, 0.25, 0.5, 1, 2, and 4 hr and allowed to clot. Serum was harvested and stored at -20 C until tested. Serum concentrations of DMP and EON were determined by the cup-plate method using 1.75% bovine albumin as the diluent and *Sarcina lutea* as the test organism. These tests were performed by Bristol Laboratories, Syracuse, N.Y. The serum concentration of penicillin V was determined by the same method in our laboratory. Serum antimicrobial activity was determined in parallel, using Trypticase soy broth and 50% serum as diluents. Penicillin G-sensitive (I.D. White) and resistant (I.D. 76) strains of staphylococci were used as indicator organisms in the performance of these tests on sera from the first crossover study. The penicillin G-sensitive strain of staphylococcus and a Viridans streptococcus (I.D. 122) were used as indicator organisms in the second study.

## Results

The efficiency of the nine penicillins in the various protein media is presented in Table 1. As the concentration of bovine

TABLE 1. *Efficiency of nine penicillins in various protein media**

| Penicillins | Minimal inhibitory concentration (μg/ml) | | | | | Efficiency | | | |
|---|---|---|---|---|---|---|---|---|---|
| | Trypticase soy broth | 1.75% Bovine albumin | 7% Bovine albumin | 50% Pooled serum | 100% Pooled serum | 1.75% Bovine albumin | 7% Bovine albumin | 50% Pooled serum | 100% Pooled serum |
| | | | | | | % | % | % | % |
| Benzyl | 0.021 | 0.028 | 0.055 | 0.043 | 0.079 | 75.0 | 38.2 | 49.0 | 26.6 |
| Phenylmercaptomethyl | 0.008 | 0.012 | 0.055 | 0.043 | 0.055 | 66.7 | 14.5 | 18.8 | 14.5 |
| Phenoxymethyl | 0.020 | 0.024 | 0.065 | 0.082 | 0.082 | 83.3 | 30.8 | 24.5 | 24.4 |
| alpha-Phenoxyethyl | 0.024 | 0.037 | 0.130 | 0.097 | 0.098 | 64.9 | 18.5 | 24.7 | 24.5 |
| alpha-Phenoxypropyl | 0.041 | 0.130 | 0.590 | 0.390 | 0.680 | 31.5 | 6.9 | 10.5 | 6.0 |
| alpha-Phenoxyisobutyl | 0.175 | 0.585 | 2.186 | 0.623 | 2.186 | 29.9 | 8.0 | 28.1 | 8.0 |
| 2,6-Dimethoxyphenyl | 0.936 | 0.975 | 1.873 | 1.124 | 1.092 | 96.0 | 50.0 | 83.3 | 85.7 |
| 5-Methyl-3-phenyl-4-isoxozolyl | 0.156 | 0.224 | 0.858 | 1.248 | 2.03 | 69.6 | 18.2 | 12.5 | 7.68 |
| 2-Ethoxy-1-naphthyl | 0.175 | 2.05 | 3.437 | 0.897 | 1.25 | 8.5 | 5.1 | 19.5 | 14.0 |

*Results are the average of 3 to 7 determinations for each penicillin utilizing a *Staphylococcus aureus* (ID White), inoculum $9 \times 10^5$, as the test organism.

albumin was increased there was a decrease in efficiency of each of the penicillins. DMP was the least affected by bovine albumin while POP, POIB, and EON exhibited a marked decrease in activity in this test system. The results with 50% and whole pooled human serum roughly parallel those observed with bovine albumin. Direct comparison of the two systems cannot be made since bovine and human albumin are different proteins and the concentration of albumin is not the same in the two systems. In human serum, DMP was most efficient while POP, MPI, and POIB were markedly inefficient. It is apparent that penicillins V, POE, and DMP had the same activity in both 50% and undiluted serum, indicating that 50% or possibly a lesser concentration of human serum may result in maximal interference of activity for these agents. The remaining agents were less efficient in whole serum than in the 50% system.

Although data are not complete, the loss of antimicrobial activity demonstrated here correlates closely with the degree of protein binding of the various penicillins demonstrated by Tompsett and coworkers (1947) and Kunin (1961). These data also compare favorably with those of Gourevitch et al. (1961) where applicable.

The results of the crossover study employing penicillins DMP, MPI, and V are presented in Fig. 1 and 2. Figure 1 depicts results with the nonpenicillinase-producing strain of staphylococcus as the test organism. In Fig. 1, it is apparent that the serum inhibitory activity, when determined in the 50% pooled human serum system, is less for each penicillin than when broth is employed as the diluent. The discrepancy is greatest for MPI, intermediate for V, and least for DMP. Comparison of serum concentration and serum antibacterial activity indicates that approximately 93% of DMP, 11% of MPI, and 17% of V remained biologically active in the presence of 50% serum. These data correlate closely with the efficiency of these agents as illustrated in Table 1. It is also apparent that, although penicillin V produced the lowest serum concentrations of the

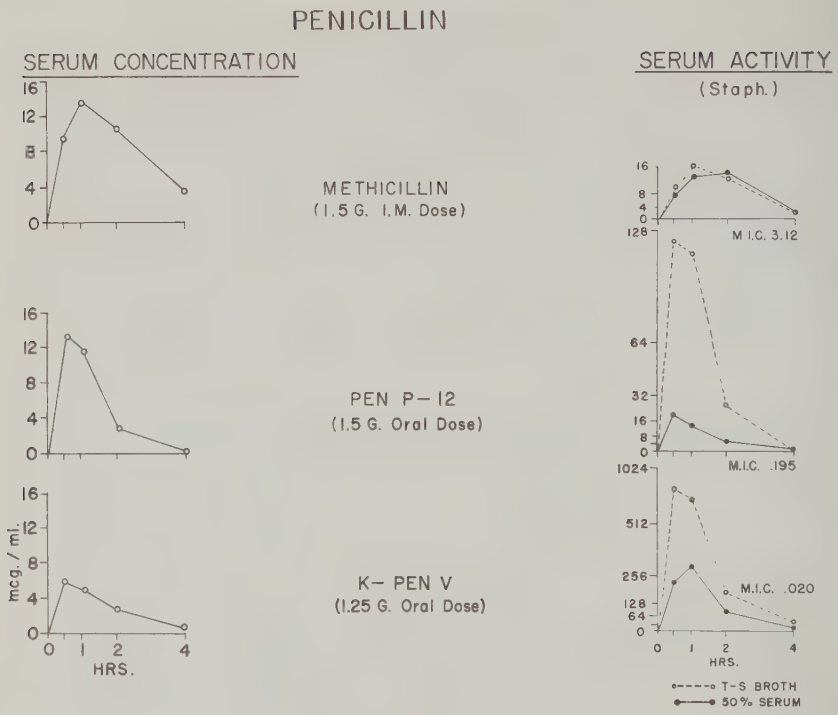


FIG. 1. *The average serum concentration and serum inhibitory activity after a single fasting dose of methicillin (DMP), penicillin P-12 (MPI), and penicillin V in a crossover study in ten normal adults. Concentration assayed by the Sarcina lutea cup-plate method utilizing 1.75% bovine albumin as the reference standard and serum diluent. Serum activity, expressed as the reciprocal of the maximal inhibiting dilution, for a nonpencillinase-producing Staphylococcus aureus (ID White) at an average inoculum of* $2 \times 10^6$. *Serum diluted in Trypticase soy broth (T-S Broth) and 50% pooled human serum.*

three agents, the resultant serum antimicrobial activity of this penicillin was greater than either of the others. This is a reflection of the lesser activity of DMP on a weight basis and the marked loss of activity of MPI in the presence of serum when compared to penicillin V, and illustrates the importance of considering both of these variables in the evaluation of an antimicrobial agent.

Figure 2 depicts results when the same three sets of sera were tested using a penicillinase-producing strain of staphylococcus as the test organism. Again, in 50% serum, the antistaphylococcal activity of MPI was considerably less than when determined in a serum-free medium, and DMP remained little affected by the presence of serum.

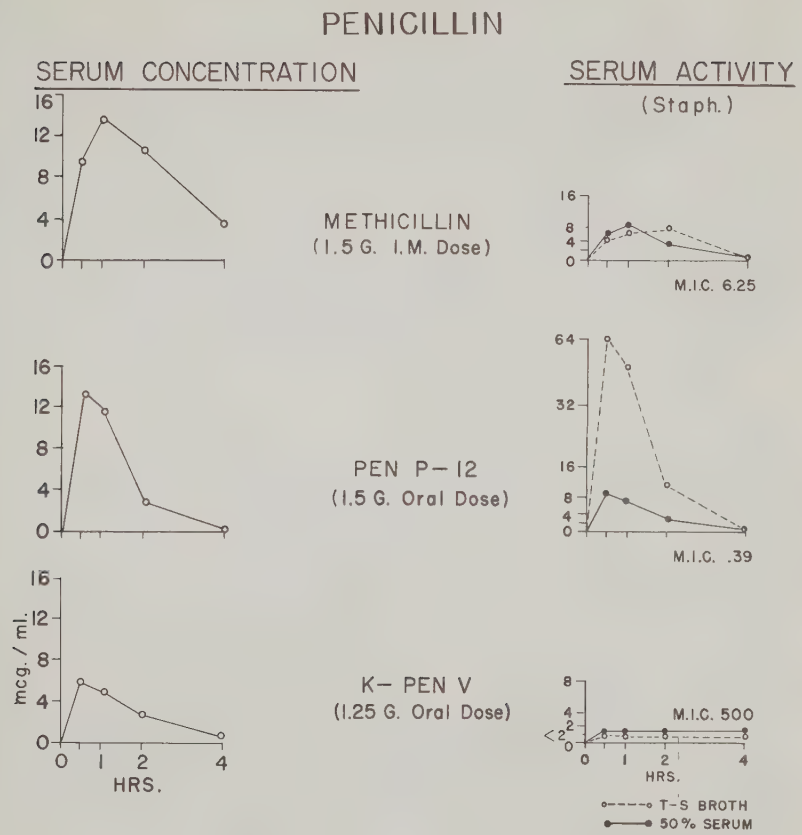


FIG. 2. *Activity of the sera illustrated in Fig. 1 for a penicillinase-producing Staphylococcus aureus (ID 76) at an average inoculum of* $1 \times 10^6$.

In sharp contrast to the results obtained with a nonpenicillinase-producing staphylococcus, where penicillin V produced the greatest antimicrobial effect, this agent failed to produce any detectable antimicrobial activity against this test organism.

Figure 3 illustrates the results of a similar single-dose crossover study comparing orally administered penicillins V and POP and intramuscular penicillin G, employing the nonpenicillinase-producing staphylococcus and Viridans streptococcus as test organisms. It is again apparent that the presence of 50% serum affected the resultant serum antimicrobial activity of each agent, and that this effect was most marked in the instance of POP. Of particular interest is the observation that under these conditions penicillin V produced greater

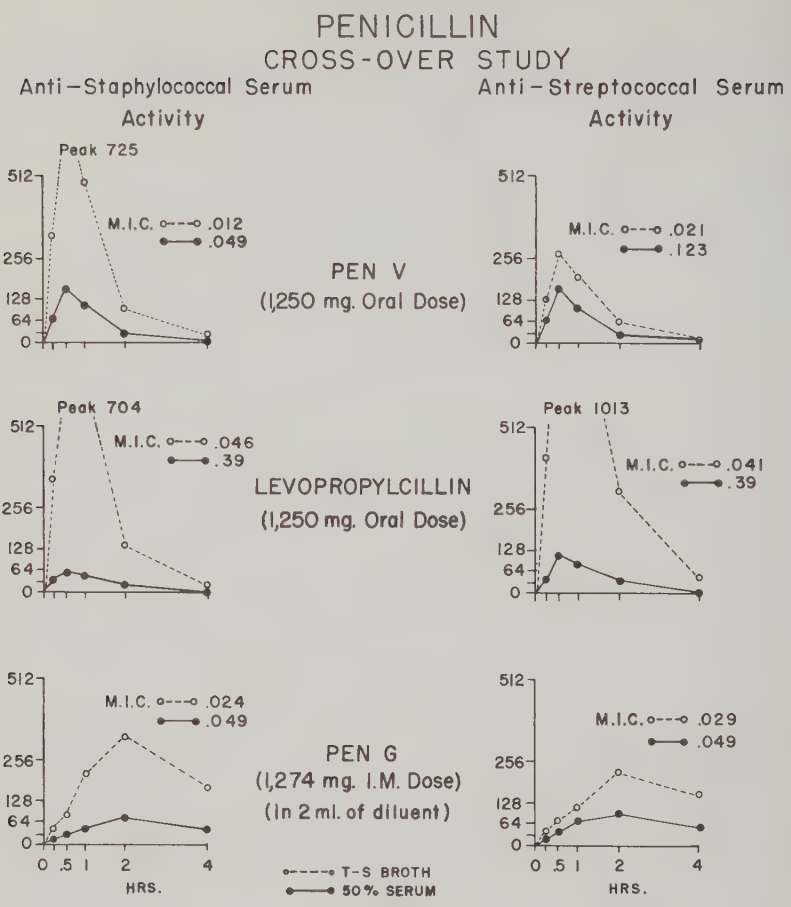


FIG. 3. *The average serum inhibitory activity in a single fasting dose crossover study in 10 normal adults given penicillin V, levopropylcillin (POP), and penicillin G. Serum activity for Staphylococcus aureus (ID White) and Viridans streptococcus (ID 122) at an average inoculum of* $3.45 \times 10^6$ *and* $4.76 \times 10^6$, *respectively.*

peak antistaphylococcal and antistreptococcal effect than either ingested POP or intramuscular crystalline penicillin G. The relatively low peak levels at 2 hr, which were only slightly less at 4 hr after a single intramuscular injection of crystalline penicillin G, were unexpected and do not agree with the observations of Tucker and Eagle (1948) concerning serum concentration of crystalline penicillin G after intramuscular administration of large doses of this agent. These authors and others (McDermott et al., 1945) observed

high peak concentrations at 30 min with a rapid decline to low levels at 4 hr. It was noted that Tucker and Eagle (1948) employed a concentration of $1 \times 10^5$ units per ml, so that 12 ml were injected in the subjects who received $1.2 \times 10^6$ units, whereas our subjects received aqueous crystalline penicillin at a concentration of $1 \times 10^6$ units per ml. Two of our subjects have subsequently received $1 \times 10^6$ units of crystalline penicillin intramuscularly in volumes of 1.5 and 10 ml. Results indicate that the more dilute solution produced a more rapid rise in serum concentration followed by a more rapid decrease in serum concentration. These results suggest that highly concentrated solutions of crystalline penicillin G may act as repository type penicillins, but more data are needed before such a statement can be made with any degree of certainty.

## Discussion

It was not the intention, nor has an attempt been made here, to compare each of the nine penicillins with the object of selecting a single superior penicillin. Rather, these data serve to re-emphasize the observations of other investigators that, in the evaluation of antimicrobial agents, it is of importance to consider not only the degree of activity, absorption, serum concentration, and excretion of the agent but also the effect of the presence of serum on the antimicrobial activity of the compounds.

As an example of these multiple factors affecting the net activity of an agent, it is useful to compare MPI and DMP. In vitro, MPI is approximately eight times more active than DMP, and this results in peak serum concentrations after oral administration that are comparable to those observed following parenteral administration of DMP. However, these obvious advantages of MPI appear to be lost due to the striking decrease of activity of this agent in the presence of serum. Similarly, POP, which has the same activity as penicillin V, appears to produce higher serum concentrations but the net antibacterial effect in the presence of serum is considerably less than that of penicillin V.

Davis (1943) and others have postulated and indirectly shown that the protein-bound fraction of an antimicrobial agent is biologically inactive. Although direct determinations of protein binding have not been performed in the present work, the degree of serum interference demonstrated in these studies correlates closely with the degree of protein binding as determined by others (Tompsett et al., 1947; Kunin, 1961) where comparable data are available, and suggests that this phenomenon is responsible for the results we have obtained.

Protein binding of antimicrobial substances is presumably reversible. It has also been shown by Davis (1943) that for sulfonamides

the per cent of binding is inversely related to the concentration of the antimicrobial agents.

It can be argued, in the instance of reversible protein binding, that as serum concentration falls, dissociation of the protein-antimicrobial complex would occur and the agent would then be able to exert its antimicrobial effect. However, as demonstrated in this investigation and by others (Davis, 1943; Tompsett et al., 1947; Gourevitch et al., 1961; Kunin, 1961), the avidity of protein for the various sulfonamides and penicillins is variable from one agent to another. In the instance of a very highly bound agent, it is conceivable that the amount of free antimicrobial agent, and thus that amount available for diffusion through semipermeable membranes to the tissues, would be so low as to lack therapeutic effect. Two other factors must also be considered: thermal and tissue inactivation. It is well known that the various penicillins are heat labile. Unpublished experiments in our laboratory have indicated that serum solutions of the crystalline penicillins reported in this study lose most, if not all, of their activity after 24 hr incubation at 37 C. However, since crystalline penicillin is so rapidly absorbed and excreted, the factor of thermal inactivation would not seem to be of great importance. On the other hand, Spitzy (1955) demonstrated in vitro that guinea pig liver slices quite rapidly inactivated certain penicillins. He noted that the various penicillins tested exhibited differing rates of inactivation. Thus, penicillins G, V, and PMM were inactivated with increasing rapidity. Spitzy also showed that there was a linear relationship between penicillin inactivation and cellular metabolism. In this connection it is interesting to speculate on the present observations that, although intramuscular DMP and orally administered MPI resulted in similar peak serum concentrations of the respective agents, serum concentration of MPI fell more rapidly than DMP. Also, significantly lesser amounts of MPI, 19% compared to 50% for DMP, were excreted in the urine (Colville and Quinn, *unpublished data*). These data suggest that MPI may be more rapidly inactivated than DMP at the tissue level. At the moment, the above considerations remain speculative and lack experimental confirmation.

Considering the point that the proportion of protein binding is inversely related to the concentration of the agent, it can be seen, even in the case of a highly bound agent, that if a great enough serum concentration could be obtained through frequent large dosing, the effect of protein interference and tissue inactivation might be offset. It follows that agents which are highly bound and are rapidly inactivated by the tissues should be administered frequently and in relatively large doses.

The foregoing discussion has been presented to indicate that knowledge of the fate of an antimicrobial agent in vivo is largely incomplete. It becomes apparent that reliance on determination of

serum concentration and antimicrobial activity of any given antimicrobial agent by conventional methods is at best a crude index of the potential therapeutic efficacy of the agent. In lieu of more precise testing methods, it behooves the clinical investigator to carefully equate the actual effect of these agents in disease states.

## Acknowledgments

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## Literature Cited

Batchelor, F. R., F. B. Doyle, J. H. C. Nayler, and G. N. Rolinson. 1959. Synthesis of penicillin: 6 aminopenicillanic acid in penicillin fermentations. Nature **183**:257-258.

Coghill, R. D., A. E. Osterberg, and G. R. Hazel. 1946. The relative effectiveness of pure penicillins G, X, and K. Science **103**:709-710.

Committee on Medical Research. U. S. Public Health Service and Food and Drug Administration. 1946. The changing character of commercial penicillin. J. Am. Med. Assoc. **131**:271-275.

Cronk, G. A., D. E. Naumann, H. Albright, and W. B. Wheatley. 1960. Laboratory and clinical studies with potassium penicillin-152 potassium (alpha phenoxyethyl) penicillin. Antibiotics Ann. 1959-1960, p. 133-145.

Davis, B. D. 1943. The binding of sulfonamide drugs by plasma proteins. A factor in determining the distribution of drugs in the body. J. Clin. Invest. **22**:753-761.

Eagle, H., and A. Musselman. 1946. The low therapeutic activity of penicillin K relative to that of penicillins F, G, and X, and its pharmacological basis. Science **103**:618-620.

Griffith, R. S. 1960. Comparison of antibiotic activity in sera after the administration of three different penicillins. Antibiotic Med. & Clin. Therapy **7**:129-135.

Gourevitch, A., G. A. Hunt, J. R. Luttinger, C. C. Cormak, and J. Lein. 1961. Activity of alpha phenoxyalkyl penicillins against sensitive and resistant staphylococci. Proc. Soc. Exptl. Biol. Med. **107**:455-458.

Grove, D. C. and W. A. Randall. 1955. Assay methods of antibiotics, a laboratory manual, p. 190-192. Medical Encyclopedia, Inc., New York.

Hobby, G. L., B. Burkhart, and B. Hyman. 1946. Chemotherapeutic action of various forms of penicillin on hemolytic streptococcal infections in mice. Proc. Soc. Exptl. Biol. Med. **63**:296-301.

Knudson, E. T., and G. N. Rolinson. 1959. Absorption and excretion studies of potassium salt of 6 (alpha propionamido) penicillanic acid. Brit. Med. J. **2**:1105-1109.

Kunin, C. M. 1961. Serum binding and excretion of four penicillin analogues following intravenous injection in man. Proc. Soc. Exptl. Biol. Med. **107**:337-341.

McCarthy, C. G., and M. Finland. 1960. Absorption and excretion of four penicillins. New Engl. J. Med. **263**:315-326.

McCarthy, C. G., H. A. Hirsh, and M. Finland. 1960. Serum levels after a single oral dose of 6-(alpha phenoxypropionamido) penicillinate and penicillin V. Proc. Soc. Exptl. Biol. Med. **103**: 177-180.

McCarthy, C. G., G. Wallmark, and M. Finland. 1961. In vitro activity of various penicillins. Am. J. Med. Sci. **241**:143-159.

McDermott, W., P. A. Bunn, M. Benoit, R. DuBois, and W. Hayes. 1945. Oral penicillin. Science **101**:228-229.

Spitzy, H. H. 1955. Inactivation of penicillins in the body. Antibiotics Ann. 1955-1956, p. 549-553.

Tompsett, R., S. Shultz, and W. McDermott. 1947. The relation of protein binding to the pharmacology and antibacterial activity of penicillins X, G, dihydro F, and K. J. Bacteriol. 53: 581-595.

Tucker, H. A., and H. Eagle. 1948. Serum concentrations of penicillin G in man following intramuscular injection in aqueous solution and in peanut oil-beeswax suspension. Am. J. Med. 4:343-354.

# IN VITRO EVALUATION OF SEMISYNTHETIC PENICILLINS WY-3206 AND WY- 3277

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Investigation of the antibacterial activity of semisynthetic penicillins, prepared by the reaction of 6-aminopenicillanic acid with various acylating agents, showed the high activity of Wy-3206 [6-(2-methoxy-1-naphthamido) penicillanic acid] and Wy-3277 [6-(2-ethoxy-1-naphthamido) penicillanic acid] against staphylococci resistant to potassium penicillin G. The chemical structure of these new agents and their relationship to methicillin [6-(2,6-dimethoxybenzamido) penicillanic acid] are shown in Fig. 1. This communication reports experiments relating to antibacterial activities and especially bactericidal properties. Comparisons have been made with methicillin.

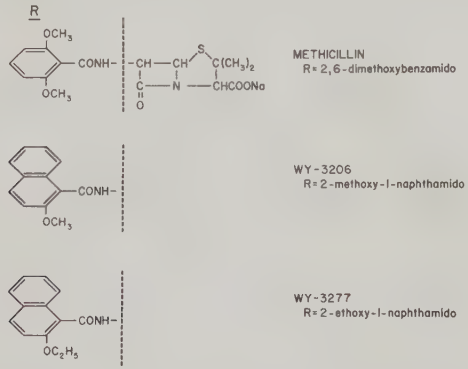


FIG. 1. *Chemical structures of new penicillins and their relationship to methicillin.*

## Materials and Methods

*Penicillins.* The potassium salts of Wy-3206 and Wy-3277 were prepared by the Medicinal Chemistry Section, Research Division, Wyeth Laboratories, Inc., Radnor, Pa. The methicillin used was a commercial lot of the sodium salt (Bristol Laboratories, Syracuse, N.Y.).

*Penicillin-resistant staphylococci.* All the cultures studied were clinical isolates that produced coagulase and penicillinase. The resistance to potassium penicillin G varied from 1.95 to more than 250 μg/ml. The naturally occurring methicillin-resistant cultures had been isolated by Jevons (1961).

*Sensitivity tests.* Minimal inhibitory concentrations were determined in brain heart infusion supplied by Baltimore Biological Laboratory, Baltimore, Md. (BBL), using the technique described by Knox (1960). Dilutions of $10^{-1}$ to $10^{-5}$ were employed in the study of the effect of inoculum size. In determining the influence of human serum, the media were so formulated that the percentage of each ingredient remained unchanged; the culture studied was *Staphylococcus aureus* strain Tex 2, resistant to more than 250 μg of potassium penicillin G.

*Effect on growth.* The general procedure followed was reported by Rolinson et al. (1960) with the following modifications: Trypticase soy broth (BBL) was substituted, the turbidities were measured in a Klett-Summerson colorimeter, 420 A, and the organism was *S. aureus* strain Tex 2.

*Bactericidal tests.* Penicillin-sensitive *S. aureus* strain Smith and penicillin-resistant *S. aureus* strain CHP were grown for 18 hr at 37 C in brain heart infusion. The cultures were diluted to $10^{-2}$ with broth, and 24-ml volumes were transferred to sterile flasks containing 1 ml of the penicillin solutions at 250 and 2,500 μg/ml (equivalent to final concentrations of 10 and 100 μg/ml). The flasks were placed in a water bath for 23 days, and samples were removed at intervals and counted. Initial washing of cells with sterile broth, prior to plating, was not necessary.

*Development of resistance.* Increasing concentrations of the penicillins incorporated in 5% peptone (Difco) were dispensed into tubes in 10-ml quantities. The tubes were inoculated with 0.1 ml of an 18-hr culture of *S. aureus* strain Smith or strain CHP. Transfers were made after 24 hr to the next higher concentration or to the same concentration for a total of ten passages.

*Serum binding.* The dialysis procedure used was that described by Anderson et al. (1955). Each penicillin was bio-assayed against its own standard and the resultant data calculated according to the formula of Smith, Levin, and Embody (1957). Potassium penicillin G was included as a control.

*Stability to staphylococcal penicillinase.* *S. aureus* strain T8234, resistant to more than 250 μg of potassium penicillin G, was grown in nutrient broth (BBL) on a shaker for 6 hr at 37 C. Wy-3277 was then added at a final concentration of 0.5 μg/ml as a penicillinase inducer and the incubation continued for an additional 18 hr; 5-ml quantities of the culture were transferred to tubes containing an equal volume of the penicillins. The final concentration of each drug was 1 mg/ml. The tubes were placed in a water bath and samples were withdrawn at intervals for bio-assay.

## Results

*Antibacterial spectrum.* Wy-3206 and Wy-3277 exhibited an overall activity similar to methicillin (Table 1). The new agents were usually more effective than methicillin in the inhibition of *S. aureus*, *Diplococcus pneumoniae*, *Streptococcus pyogenes*, *Gaffyka tetragena*, and *Klebsiella pneumoniae;* Wy-3277 was usually more active than Wy-3206.

*Sensitivity of penicillin-resistant staphylococci.* The sensitivity of strains of penicillin-resistant staphylococci is presented in Table 2. The activity of the new drugs, as with methicillin, was independent of the resistance to potassium penicillin G. Both Wy-3206 and Wy-3277 were more active than methicillin and the general order of activity was in a ratio of 4:1.

*Sensitivity of methicillin-resistant staphylococci.* Penicillins Wy-3206 and Wy-3277 were generally more effective than methicillin against the naturally occurring methicillin-resistant strains (Table 3), but the differences were not significant. Larger quantities of each penicillin were required for inhibition of strains naturally resistant to methicillin than strains resistant to potassium penicillin G (7.8 to 31.25 vs. 0.244 to 1.95 μg/ml).

*Effect of inoculum size.* The size of the inoculum of a penicillin-sensitive culture (*S. aureus* strain 209P) had less effect on the minimal inhibitory concentration of Wy-3277 than on the other penicillins tested (Table 4). When a penicillin-resistant culture (*S. aureus* strain CHP) was used, the effect was twofold with methicillin and fourfold with Wy-3206 and Wy-3277.

*Effect on growth.* It was shown that the growth rate of a penicillin-resistant culture decreased progressively as the concentration of the penicillins was increased (Fig. 2). Wy-3206 and Wy-3277 at levels of 1 μg/ml inhibited the organism as effectively as methicillin at 2.0 μg/ml, and Wy-3277 at 0.2 μg/ml was as inhibitory as methicillin and Wy-3206 at 0.5 μg/ml.

*Bactericidal tests.* Cultures of *S. aureus* strain Smith were sterile after 48 hr of contact with potassium penicillin G, Wy-3206, and Wy-3277 at a level of 10 μg/ml (Fig. 3). The killing rate with the same concentration of methicillin was initially more rapid but 72 hr were required for complete sterilization (Fig. 3). Complete killing was obtained with all the agents at a more rapid rate (Fig. 4) when the concentration was increased to 100 μg/ml. The rate with methicillin was again slower and was comparable to that obtained with the other penicillins at 10 μg/ml.

Prolonged incubation with the penicillin-resistant *S. aureus* strain CHP, utilizing the drugs at a level of 10 μg/ml, resulted in sterilization with Wy-3277, approximately 99% diminution with Wy-3206, and a "climb up" with methicillin (Fig. 5). When the concentration employed was 100 μg/ml, there was sterilization by Wy-3277 and

TABLE 1. *Antibacterial spectrum of methicillin, Wy-3206, and Wy-3277*

| Organism | Strain | Minimal inhibitory concentration | | |
|---|---|---|---|---|
| | | Methicillin | Wy-3206 | Wy-3277 |
| | | *μg/ml* | *μg/ml* | *μg/ml* |
| *Aerobacter aerogenes* | Wy | 62.5 | 7.81 | 15.6 |
| *Alcaligenes faecalis* | H841 | 250 | 250 | 250 |
| *Alcaligenes faecalis* | Wy-129 | 125 | 250 | 250 |
| *Alcaligenes faecalis* | H842 | 250 | 250 | 250 |
| *Bacillus cereus* | 10987 | 31.2 | 125 | 250 |
| *Bacillus sphaericus* var. *fusiformis* | 12300 | 3.90 | 1.95 | 3.90 |
| *Bacillus mycoides* | 4I26 | 0.122 | 0.122 | 0.244 |
| *Bacillus subtilis* | 6633 | 0.122 | 0.244 | 0.488 |
| *Bacillus subtilis* | B972 | 62.5 | 250 | 250 |
| *Bordetella bronchiseptica* | 4617 | 250 | 250 | 250 |
| *Corynebacterium xerosis* | 11036 | 1.95 | 1.95 | 1.95 |
| *Diplococcus pneumoniae* type I* | 37 | 0.244 | 0.122 | 0.061 |
| *Diplococcus pneumoniae* type I* | | 0.244 | 0.061 | 0.031 |
| *Diplococcus pneumoniae** | And | 0.488 | 0.122 | 0.122 |
| *Escherichia coli* | 5905-15-5 | 250 | 250 | 250 |
| *Escherichia coli* | 4I67 | 250 | 250 | 250 |
| *Escherichia coli* | 6880 | 250 | 250 | 250 |
| *Escherichia coli* | SR | 250 | 250 | 250 |
| *Gaffyka tetragena* | 10875 | 0.976 | 0.488 | 0.244 |
| *Klebsiella pneumoniae* | Wy | 125 | 62.5 | 15.6 |
| *Klebsiella pneumoniae* | 10031 | 62.5 | 62.5 | 15.6 |
| *Klebsiella pneumoniae* | Yale | 250 | 250 | 250 |
| *Klebsiella pneumoniae* | KmR | 250 | 250 | 250 |
| *Micrococcus flavus* | 4I23 | 3.90 | 0.976 | 0.488 |
| *Neisseria catarrhalis* | Wy | 31.3 | 7.81 | 3.90 |
| *Neisseria catarrhalis* | 8192 | 0.976 | 0.244 | 0.488 |
| *Proteus vulgaris* | 6896 | 250 | 250 | 250 |
| *Proteus vulgaris* | 4I66 | 250 | 250 | 250 |
| *Proteus vulgaris* | 9920 | 15.6 | 250 | 250 |
| *Pseudomonas aeruginosa* | Wy | 250 | 250 | 250 |
| *Salmonella paratyphi* type B | | 31.3 | 31.3 | 15.6 |
| *Salmonella typhosa* | SaD12 | 250 | 250 | 250 |
| *Sarcina lutea* | 9341 | 0.061 | 0.031 | 0.0152 |
| *Staphylococcus aureus* | 209P | 1.95 | 0.244 | 0.244 |
| *Staphylococcus aureus* | Smith | 1.95 | 0.488 | 0.488 |
| *Staphylococcus aureus* | GFF | 1.95 | 0.976 | 0.488 |
| *Staphylococcus aureus* | GF7 | 1.95 | 0.976 | 0.488 |
| *Staphylococcus aureus* | GF8 | 1.95 | 0.976 | 0.488 |
| *Staphylococcus aureus* | GF9 | 1.95 | 0.488 | 0.244 |
| *Staphylococcus aureus* | G39 | 1.95 | 0.976 | 0.488 |
| *Staphylococcus aureus* | G45 | 1.95 | 0.488 | 0.488 |
| *Staphylococcus aureus* | G50 | 1.95 | 0.976 | 0.488 |
| *Staphylococcus aureus* | G55 | 7.81 | 3.90 | 0.488 |
| *Staphylococcus aureus* | G80 | 3.90 | 0.976 | 0.488 |
| *Staphylococcus aureus* | G82 | 3.90 | 0.488 | 0.488 |
| *Staphylococcus aureus* | J124 | 1.95 | 0.488 | 0.244 |
| *Staphylococcus aureus* | J357 | 3.90 | 0.488 | 0.488 |
| *Staphylococcus aureus* | J1187 | 1.95 | 0.488 | 0.488 |
| *Streptococcus faecalis** | 10541 | 250 | 31.3 | 62.5 |
| *Streptococcus pyogenes** | T93 | 0.244 | 0.061 | 0.031 |
| *Streptococcus pyogenes** | GrA | 0.244 | 0.061 | 0.031 |
| *Streptococcus pyogenes** | 8668 | 1.95 | 0.488 | 0.488 |
| *Streptococcus pyogenes** | 46 | 0.244 | 0.061 | 0.031 |
| *Streptococcus pyogenes** | 12384 | 0.488 | 0.122 | 0.061 |
| *Streptococcus pyogenes** | A | 0.488 | 0.061 | 0.031 |
| *Streptococcus pyogenes** | 48 | 0.122 | 0.031 | 0.061 |
| *Streptococcus pyogenes** | Type D | 62.5 | 31.3 | 7.81 |
| *Streptococcus mitis* | | 0.061 | 0.031 | 0.0152 |

*Medium + 5% human serum.

TABLE 2. *Comparison of sensitivity of clinical strains of penicillin-resistant staphylococci to potassium penicillin G, methicillin, Wy-3206, and Wy-3277*

| Strain | Minimal inhibitory concentration | | | |
|---|---|---|---|---|
| | Potassium penicillin G | Methicillin | Wy-3206 | Wy-3277 |
| | μg/ml | μg/ml | μg/ml | μg/ml |
| CHP | 15.6 | 7.81 | 1.95 | 1.95 |
| 2914 | 1.95 | 3.90 | 0.976 | 0.488 |
| 2920 | 250 | 3.90 | 1.95 | 0.976 |
| 2921 | 250 | 3.90 | 0.976 | 0.976 |
| 2922 | 3.90 | 3.90 | 1.95 | 0.976 |
| 2928 | 250 | 3.90 | 0.976 | 0.976 |
| 2942A | 250 | 3.90 | 0.976 | 0.976 |
| 2942B | >250 | 3.90 | 0.976 | 0.488 |
| 2950 | 250 | 3.90 | 1.95 | 0.976 |
| 2967 | 3.90 | 1.95 | 0.976 | 0.488 |
| 53-180 | >250 | 3.90 | 1.95 | 0.976 |
| J 2 | 3.90 | 1.95 | 0.488 | 0.488 |
| J 144 | 125 | 1.95 | 0.488 | 0.488 |
| J 241 | 1.95 | 0.976 | 0.244 | 0.244 |
| LA 105 | 250 | 3.90 | 1.95 | 0.976 |
| LA 108 | >250 | 3.90 | 1.95 | 0.976 |
| LA 418 | >250 | 3.90 | 1.95 | 0.976 |
| LA 459 | 31.25 | 3.90 | 0.976 | 0.976 |
| LA 534 | 31.25 | 3.90 | 0.976 | 0.976 |
| LA 671 | 31.25 | 3.90 | 1.95 | 0.976 |
| LA 973 | >250 | 3.90 | 0.976 | 0.976 |
| T 8070 | 250 | 3.90 | 0.976 | 0.976 |
| T 8234 | >250 | 3.90 | 0.976 | 0.976 |
| T 8705 | >250 | 3.90 | 1.95 | 0.488 |
| T 8740 | >250 | 3.90 | 0.976 | 0.488 |
| T 9006 | 250 | 3.90 | 0.976 | 0.488 |
| T 9267 | >250 | 3.90 | 0.976 | 0.488 |
| T 9763 | 250 | 3.90 | 0.976 | 0.976 |
| Tex 1 | >250 | 3.90 | 0.976 | 0.488 |
| Tex 2 | >250 | 3.90 | 0.976 | 0.976 |
| Tex 3 | >250 | 3.90 | 0.976 | 0.488 |
| Tex 4 | >250 | 3.90 | 0.976 | 0.976 |
| Tex 5 | >250 | 3.90 | 0.976 | 0.488 |
| Tex 7 | >250 | 3.90 | 0.976 | 0.976 |
| Tex 9 | 250 | 3.90 | 1.95 | 0.976 |

TABLE 3. *Penicillin sensitivity of naturally occuring methicillin-resistant strains of Staphylococcus aureus*

| Strain | Minimal inhibitory concentration | | | |
|---|---|---|---|---|
| | Potassium penicillin G | Methicillin | Wy-3206 | Wy-3277 |
| | μg/ml | μg/ml | μg/ml | μg/ml |
| 60/13136 | 62.5 | 31.25 | 15.6 | 15.6 |
| 60/13137 | 62.5 | 31.25 | 7.81 | 31.25 |
| 60/10395 | 62.5 | 15.6 | 7.81 | 15.6 |
| 60/14668 | 31.25 | 31.25 | 15.6 | 31.25 |
| 61/22 | 31.25 | 7.81 | 7.81 | 15.6 |
| 61/763 | 62.5 | 31.25 | 7.81 | 15.6 |
| 61/498 | 31.25 | 15.6 | 7.81 | 7.81 |

TABLE 4. *Effect of inoculum size of penicillin-sensitive and penicillin-resistant strains of Staphylococcus aureus on minimal inhibitory concentrations of penicillins*

| Strain | Inoculum | Minimal inhibitory concentration | | |
|---|---|---|---|---|
| | | Methicillin | Wy-3206 | Wy-3277 |
| | *no. organisms* | *μg/ml* | *μg/ml* | *μg/ml* |
| 209P (sensitive) | $2.52 \times 10^6$ | 1.95 | 0.488 | 0.244 |
| | $2.52 \times 10^5$ | 1.95 | 0.244 | 0.244 |
| | $2.52 \times 10^4$ | 0.976 | 0.244 | 0.244 |
| | $2.52 \times 10^3$ | 0.976 | 0.244 | 0.244 |
| | $2.52 \times 10^2$ | 0.488 | 0.122 | 0.122 |
| Tex 2 (resistant) | $3.88 \times 10^6$ | 3.90 | 3.90 | 1.95 |
| | $3.88 \times 10^5$ | 1.95 | 1.95 | 0.488 |
| | $3.88 \times 10^4$ | 1.95 | 0.976 | 0.488 |
| | $3.88 \times 10^3$ | 1.95 | 0.976 | 0.488 |
| | $3.88 \times 10^2$ | 1.95 | 0.976 | 0.488 |

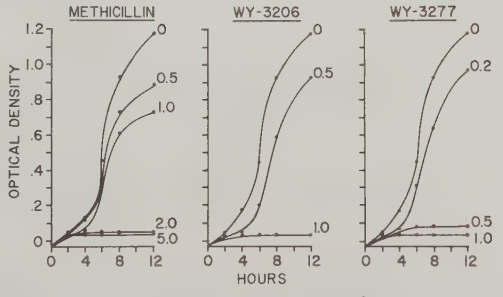


FIG. 2. *Effect of various penicillins on growth of a resistant strain of Staphylococcus aureus. Concentrations in μg/ml.*

TABLE 5. *Effect of human serum on the minimal inhibitory concentration of penicillins using a penicillin-resistant Staphylococcus aureus*

| Serum | Minimal inhibitory concentration | | |
|---|---|---|---|
| | Methicillin | Wy-3206 | Wy-3277 |
| % | *μg/ml* | *μg/ml* | *μg/ml* |
| 0 | 3.90 | 0.976 | 0.488 |
| 10 | 3.90 | 0.976 | 0.976 |
| 20 | 7.81 | 1.95 | 0.976 |
| 30 | 7.81 | 1.95 | 1.95 |
| 40 | 7.81 | 1.95 | 1.95 |
| 50 | 7.81 | 3.90 | 3.90 |

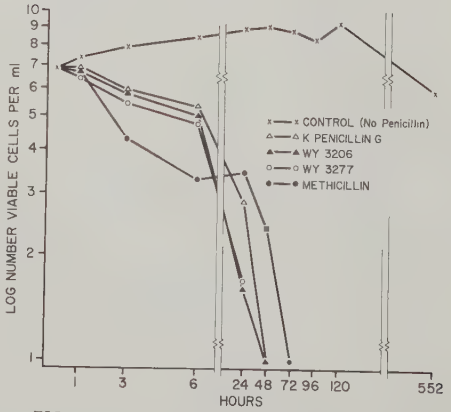


FIG. 3. *Bactericidal effect of various penicillins at 10 μg/ml on a sensitive strain of Staphylococcus aureus.*

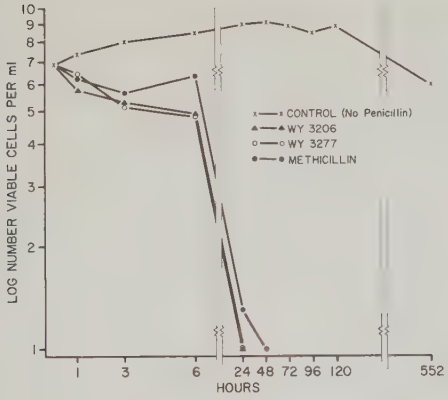


FIG. 4. *Bactericidal effect of various penicillins at 100 μg/ml on a sensitive strain of Staphylococcus aureus.*

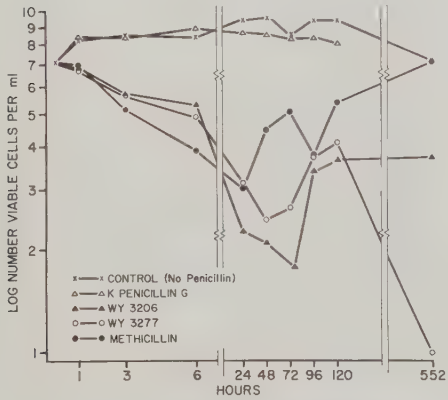


FIG. 5. *Bactericidal effect of various penicillins at 10 μg/ml on a resistant strain of Staphylococcus aureus.*

Wy-3206 in 2 and 23 days, respectively, and a "climb-up" with methicillin (Fig. 6). A similar increase in bacterial population with methicillin has been reported by Knox (1960).

*Development of resistance.* Continual transfer for ten passages of the penicillin-sensitive culture in the presence of potassium penicillin G and methicillin resulted in a 64-fold increase in tolerance (Fig. 7). The culture showed a fourfold increase in resistance with Wy-3206; no increase in tolerance appeared until the seventh transfer. Resistance to Wy-3277 developed more slowly than to methicillin and represented a tenfold increase.

A greater resistance (four to eightfold) was developed by the resistant *S. aureus* when passaged in the presence of all the penicillins. The increase in tolerance to potassium penicillin G and Wy-3206 did not occur until after the seventh transfer; the patterns of resistance for methicillin and Wy-3277 were similar (Fig. 8).

*Effect of serum on activity.* Table 5 shows no significant diminution in the activity of methicillin in the presence of human serum. There was a gradual decrease in activity of Wy-3206 and Wy-3277 with increasing concentrations of serum. In the presence of 50% serum the decrease was fourfold with Wy-3206 and eightfold with Wy-3277.

*Serum binding.* The average binding values obtained with pooled human serum for potassium penicillin G, methicillin, Wy-3206, and Wy-3277 were 55.5, 38.0, 70.6, and 79.0%, respectively.

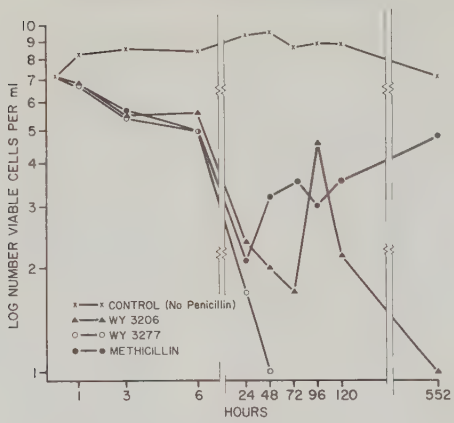


FIG. 6. *Bactericidal effect of various penicillins at 100 μg/ml on a resistant strain of Staphylococcus aureus.*

TABLE 6. *Stability of penicillins to staphylococcal penicillinase*

| Time | Residual penicillin | | | |
|---|---|---|---|---|
| | Potassium penicillin G | Methicillin | Wy-3206 | Wy-3277 |
| *min* | *units/ml* | *μg/ml* | *μg/ml* | *μg/ml* |
| 0 | 81 | 788 | 855 | 1,065 |
| 10 | 8.1 | 735 | 840 | 1,100 |
| 20 | 0 | 825 | 939 | 950 |
| 40 | 0 | 855 | 930 | 1,065 |
| 60 | 0 | 765 | 903 | 1,050 |
| Control 60 | 1,600 | 885 | 930 | 1,075 |

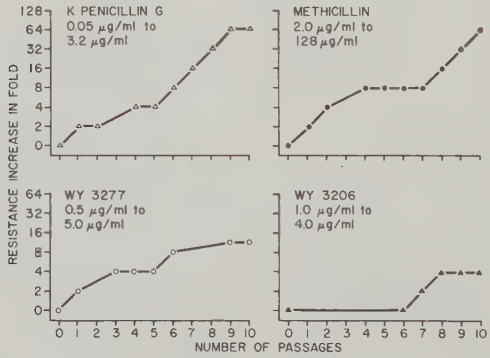


FIG. 7. *Development of resistance by a sensitive strain of Staphylococcus aureus after exposure to increasing concentrations of various penicillins.*

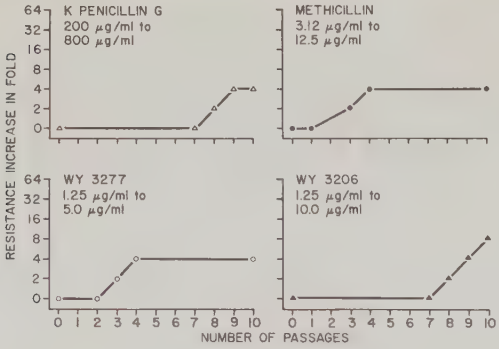


FIG. 8. *Further development of resistance by a resistant strain of Staphylococcus aureus after exposure to increasing concentrations of various penicillins.*

*Resistance to staphylococcal penicillinase.* Inactivation of the synthetic penicillins did not occur during a 60-min period. In contrast, potassium penicillin G was completely destroyed in 10 to 24 min (Table 6).

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## Literature Cited

Anderson, R. C., C. C. Lee, H. M. Worth, and K. K. Chen. 1956, Pharmacologic and toxicologic studies with penicillin V. Antibiotics Ann. 1955-1956. p. 540-548.

Jevons, M. P. 1961. Celbenin-resistant staphylococci. Brit. Med. J. 1:124-125.

Knox, R. 1960. A new penicillin (BRL 1241) active against penicillin-resistant staphylococci. Brit. Med. J. 2:690-693.

Rolinson, G. N., S. Stevens, F. R. Batchelor, J. C. Wood, and E. B. Chain. 1960. Bacteriological studies on a new penicillin - BLR 1241. Lancet 2:564-567.

Smith, C. I., J. D. Levin, and D. R. Embody. 1957. Studies of the binding of penicillins G and V by human plasma. Antibiotics Ann. 1956-1957, p. 306-310.

# THERAPEUTIC ACTIVITY OF SEMISYNTHETIC PENICILLINS WY-3206 AND WY-3277 IN EXPERIMENTAL INFECTIONS IN MICE

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The in vitro properties of semisynthetic penicillins Wy-3206 [6-(2-methoxy-1-naphthamido) penicillanic acid] and Wy-3277 [6-(2-ethoxy-1-naphthamido) penicillanic acid] were previously characterized (Rosenman and Warren, 1962). The present study supplements the in vitro data with information pertaining to chemotherapeutic activity of Wy-3206 and Wy-3277 in mice infected experimentally with several species of pathogenic organisms. Observations were made also on the fate of a potassium penicillin G-resistant strain of *Staphylococcus aureus* in the kidneys of mice after treatment with Wy-3206 and Wy-3277. Potassium penicillin G and methicillin were included in these studies as reference standards.

## Materials and Methods

Swiss albino male mice, weighing 16 to 18 g, were employed exclusively in these studies. The following organisms were injected intraperitoneally to induce infections in mice: the Smith strain of *S. aureus*, sensitive to potassium penicillin G; a clinical isolate of *S. aureus* strain CHP from the Children's Hospital of Philadelphia, naturally resistant to 250 $\mu$g/ml of potassium penicillin G; *Streptococcus pyogenes* strain C203; and *Diplococcus pneumoniae*, type I, strain 37, obtained from the Merck Institute of Therapeutic Research.

Strain virulence and concentration of inoculum of each species was maintained by storage at -70 C in a $CO_2$ chest. Cultures (18-hr) of each species, grown on appropriate solid media, were harvested with brain heart infusion (Baltimore Biological Laboratory, Baltimore, Md.), pooled in large volumes, ampouled, and quick-frozen in a $CO_2$-acetone bath for storage. Viable counts and virulence titrations in mice were performed at intervals to assure uniformity of the frozen pools. The number of viable cells in 0.5 ml of 5% gastric mucin necessary to kill 95 $\pm$ 5% of the infected mice was

determined for each species by replicate trials. The equivalent 1 $LD_{95 \pm 5}$ for each of the strains is as follows: *S. aureus* strain Smith, 200 $LD_{50}$; *S. aureus* strain CHP, 12 $LD_{50}$; *S. pyogenes* strain C203, 200 $LD_{50}$; and *D. pneumoniae*, type I, 100 $LD_{50}$.

Experimental animals were challenged intraperitoneally with the respective strains, pooled, and distributed at random into separate animal jars in groups of five mice each. Treatment with the penicillins was initiated intramuscularly within 0.5 to 1 hr of random selection. A second dose was injected approximately 6 hr later. At 24 hr postinfection all surviving animals received a third and final dose of the penicillins. Appropriate numbers of randomly selected infected controls, amounting to at least 10% of the infected pooled mice, were included in each trial. All animals were observed for a period of 14 days. Deaths were recorded daily and the 50% protective dose ($PD_{50}$) was determined by the method of Reed-Muench (1938).

The fate of the penicillin-resistant strain of *S. aureus* in the kidneys of mice treated with the four penicillins was determined as follows. Mice were challenged intraperitoneally with 1 $LD_{95 \pm 5}$ dose of the organism, pooled, and distributed at random with five animals per jar. Groups of 40 mice each were treated with a single intramuscular dose of the appropriate penicillin. The following two concentration levels were studied: 2.7 and 0.675 mg. At selected intervals, within a span of 28 days, five mice were selected at random from each group, killed, and the kidneys removed under asceptic conditions. The paired kidneys of each mouse were weighed, and sterile nutrient broth was added to give a 10% suspension, by weight, after homogenizing in a tissue grinder. The homogenized suspensions from each animal were serially diluted in cold nutrient broth and the number of viable organisms per milligram of tissue determined by the agar pour plate method.

## Results

The results compare the relative therapeutic activities of potassium penicillin G, Wy-3206, Wy-3277, and methicillin against the indicated species. These data were accumulated from replicate experiments.

In Table 1 the activities of four penicillins in mice infected with the Smith strain of *S. aureus* are compared. Although methicillin appears to be better than Wy-3206 or Wy-3277, these differences are not statistically significant. Potassium penicillin G, as expected, was significantly more active against the penicillin-sensitive strain of *S. aureus* than the other penicillins.

TABLE 1. *Therapeutic activity of Wy-3206, Wy-3277, methicillin, and potassium penicillin G in mice infected with Staphylococcus aureus strain Smith*

| Agent | Total im dose | Survivors | | $PD_{50}$* |
|---|---|---|---|---|
| | *mg/kg* | *total* | % | *mg/kg* |
| Wy-3206 | 300 | 20/20 | 100 | 21.3 ± 1.9 |
| | 150 | 20/20 | 100 | |
| | 75 | 29/30 | 97 | |
| | 37.5 | 25/30 | 83 | |
| | 18.8 | 17/40 | 43 | |
| | 9.4 | 3/40 | 8 | |
| | 4.7 | 0/40 | 0 | |
| Wy-3277 | 75 | 20/20 | 100 | 22.5 ± 1.33 |
| | 37.5 | 27/30 | 90 | |
| | 18.8 | 13/30 | 43 | |
| | 9.4 | 1/30 | 3 | |
| Methicillin | 300 | 20/20 | 100 | 17.4 ± 4.27 |
| | 150 | 20/20 | 100 | |
| | 75 | 37/40 | 93 | |
| | 37.5 | 44/50 | 88 | |
| | 18.8 | 40/60 | 68 | |
| | 9.4 | 5/60 | 8 | |
| Potassium penicillin G | 9.4 | 15/15 | 100 | |
| | 4.7 | 22/25 | 88 | 1.66 ± 0.22 |
| | 2.4 | 28/35 | 80 | |
| | 1.2 | 15/30 | 50 | |
| | 0.6 | 3/30 | 10 | |
| Untreated controls | – | 5/203 | 3 | – |

*At 14 days; mean ± survival dose.

The behavior of the four test agents is shown in mice infected with the potassium penicillin G-resistant strain of *S. aureus* (Table 2). Infected mice responded favorably to Wy-3206, Wy-3277, and methicillin, and the $PD_{50}$ value for Wy-3277 was lower than those determined for Wy-3206 or methicillin.

Table 3 summarizes the $PD_{50}$ values of the test agents in mice challenged with *S. pyogenes*. The best results were obtained with potassium penicillin G. The latter proved to be 5, 16, and 25 times more active than Wy-3277, Wy-3206, and methicillin, respectively. Wy-3277 demonstrated a significantly higher order of activity than Wy-3206 and methicillin under the same experimental conditions.

The $PD_{50}$ values compare the protection afforded by the three semisynthetic penicillins in mice infected with *D. pneumoniae*, type I (Table 4). Wy-3277 was three times and Wy-3206 was twice as effective as methicillin against this infection.

The effect of single intramuscular doses (2.7 and 0.675 mg) of the four penicillins is shown on the survival of a penicillin-resistant strain of *S. aureus* in the kidneys of mice over a period of 28 days. The results are expressed as the median number of viable organisms

TABLE 2. *The therapeutic activity of Wy-3206, Wy-3277, methicillin, and potassium penicillin G in mice infected with Staphylococcus aureus strain CHP*

| Agent | Total im dose | Survivors | | $PD_{50}$* |
|---|---|---|---|---|
| | *mg./kg.* | *total* | *%* | *mg/kg* |
| Wy-3206 | 300 | 45/45 | 100 | 24.6 ± 1.0 |
| | 150 | 43/45 | 96 | |
| | 75 | 41/45 | 91 | |
| | 37.5 | 35/45 | 78 | |
| | 18.8 | 16/45 | 36 | |
| | 9.4 | 8/25 | 32 | |
| | 4.7 | 5/20 | 25 | |
| Wy-3277 | 300 | 20/20 | 100 | 13.5 ± 5.67 |
| | 150 | 20/20 | 100 | |
| | 75 | 50/50 | 100 | |
| | 37.5 | 47/50 | 94 | |
| | 18.8 | 35/50 | 70 | |
| | 9.4 | 15/50 | 30 | |
| | 4.7 | 8/50 | 16 | |
| Methicillin | 300 | 45/45 | 100 | 20.2 ± 8.20 |
| | 150 | 43/45 | 96 | |
| | 75 | 50/55 | 91 | |
| | 37.5 | 55/65 | 85 | |
| | 18.8 | 45/75 | 60 | |
| | 9.4 | 22/55 | 40 | |
| | 4.7 | 5/50 | 8 | |
| Potassium penicillin G | 600 | 25/25 | 100 | 134.4 ± 49.4 |
| | 300 | 29/30 | 97 | |
| | 150 | 15/30 | 50 | |
| | 75 | 7/30 | 23 | |
| | 37.5 | 7/30 | 23 | |
| Untreated controls | – | 9/360 | 2.8 | – |

*At 14 days; mean ± survival dose.

TABLE 3. *The therapeutic activity of Wy-3206, Wy-3277, methicillin, and potassium penicillin G in mice infected with Streptococcus pyogenes strain C203*

| Agent | Total im dose | Survivors | | $PD_{50}$* |
|---|---|---|---|---|
| | *mg/kg* | *total* | *%* | *mg/kg* |
| Wy-3206 | 75 | 30/30 | 100 | 12.5 ± 0.8 |
| | 37.5 | 25/30 | 83 | |
| | 18.8 | 28/40 | 70 | |
| | 9.4 | 14/40 | 35 | |
| | 4.7 | 8/40 | 20 | |
| | 2.4 | 4/30 | 13 | |
| Wy-3277 | 75 | 20/20 | 100 | 4.1 ± 0.22 |
| | 37.5 | 20/20 | 100 | |
| | 18.8 | 29/30 | 97 | |
| | 9.4 | 28/30 | 93 | |
| | 4.7 | 20/30 | 67 | |
| | 2.4 | 4/20 | 20 | |
| Methicillin | 75 | 40/40 | 100 | 19.1 ± 6.66 |
| | 37.5 | 31/40 | 78 | |
| | 18.8 | 28/60 | 47 | |
| | 9.4 | 10/60 | 17 | |
| | 4.7 | 4/50 | 8 | |
| Potassium penicillin G | 9.4 | 18/20 | 90 | |
| | 4.7 | 18/20 | 90 | 0.78 ± 0.22 |
| | 2.4 | 24/30 | 80 | |
| | 1.2 | 21/30 | 70 | |
| | 0.6 | 13/30 | 43 | |
| | 0.3 | 8/30 | 27 | |
| Untreated controls | – | 5/200 | 2.5 | – |

*At 14 days; mean ± survival dose.

TABLE 4. *The therapeutic activity of Wy-3206, Wy-3277, and methicillin in mice infected with Diplococcus pneumoniae type I*

| Agent | Total dose | Survivors | | $PD_{50}$* |
|---|---|---|---|---|
| | *mg/kg* | *total* | *%* | *mg/kg* |
| Wy-3206 | 75 | 20/20 | 100 | 8.3 ± 0.56 |
| | 37.5 | 20/20 | 100 | |
| | 18.8 | 17/20 | 85 | |
| | 9.4 | 7/20 | 35 | |
| | 4.7 | 4/20 | 20 | |
| Wy-3277 | 300 | 10/10 | 100 | 5.1 ± 0.77 |
| | 150 | 10/10 | 100 | |
| | 75 | 18/20 | 90 | |
| | 37.5 | 29/30 | 97 | |
| | 18.8 | 29/30 | 97 | |
| | 9.4 | 19/20 | 95 | |
| | 4.7 | 10/20 | 50 | |
| | 2.4 | 0/10 | 0 | |
| Methicillin | 300 | 10/10 | 100 | 16.2 ± 3.33 |
| | 150 | 10/10 | 100 | |
| | 75 | 29/30 | 97 | |
| | 37.5 | 26/30 | 87 | |
| | 18.8 | 18/30 | 60 | |
| | 9.4 | 3/20 | 15 | |
| Untreated controls | – | 2/125 | 1.6 | – |

*At 14 days; mean ± survival dose.

per mg of kidney tissue; the range of counts showing the degree of variation is also presented. The results with Wy-3277 at the higher dose level showed a greater degree of efficacy and uniformity with respect to the median as well as the range (Table 5). It is apparent that the destruction rate of *S. aureus* by Wy-3277 was more rapid and more complete than with the other penicillins, which suggests the possibility that the strain was unusually susceptible to Wy-3277. The results based on the median indicate a similar trend with methicillin; however, viable cells persisted throughout the study (Table 5).

The results in Table 6 compare the effectiveness of the four penicillins at one-fourth the previous dose. Wy-3277 again appears to be better than the other penicillins on the basis of the median viable count. Moreover, partial sterilization of the kidneys and uniformly lower counts with Wy-3277 were obtained during the first 3 days of the study. In contrast, 14 days were required by methicillin to eliminate persistent organisms from the kidneys.

Graphic presentation of the data obtained from the intramuscular doses describes the results in terms of the percentage of animals that were found to have positive kidney cultures. Of the four agents studied, only Wy-3277 showed a progressive decrease in the number of mice with infected kidneys. No positive cultures were obtained after the 14th day from animals injected with 2.7 mg of Wy-3277.

TABLE 5. *Fate of Staphylococcus aureus strain CHP in kidneys of mice treated intramuscularly with a single dose (2.7 mg) of potassium penicillin G, Wy-3206, Wy-3277, and methicillin*

| Agent | Days after infection | No. animals positive (total) | Median viable count* | Range of counts* |
|---|---|---|---|---|
| Potassium penicillin G | 0.25 | 5/5 | 1,004 | 384 - 12,977 |
| | 1 | 5/5 | 118 | 15 - 1,045 |
| | 3 | 5/5 | 8 | 0.4 - 107,285 |
| | 7 | 4/5 | 1.01 | 0 - 7.1 |
| | 14 | 4/5 | 0.13 | 0 - 283,019 |
| | 21 | 1/5 | 0.0 | 0 - 0.07 |
| | 28 | 3/5 | 0.03 | 0 - 0.09 |
| Wy-3206 | 0.25 | 3/5 | 0.96 | 0.65 - 34 |
| | 1 | 5/5 | 34.0 | 11.0 - 559 |
| | 3 | 5/5 | 1.03 | 0.04 - 338 |
| | 7 | 5/5 | 2.12 | 0.06 - 25,380 |
| | 14 | 4/5 | 0.74 | 0 - 1,435 |
| | 21 | 3/5 | 0.30 | 0 - 111 |
| | 28 | 0/4 | 0.0 | 0 |
| Wy-3277 | 0.25 | 5/5 | 171 | 86 - 370 |
| | 1 | 5/5 | 15.4 | 1.8 - 94 |
| | 3 | 2/5 | 0.0 | 0 - 12 |
| | 7 | 1/5 | 0.0 | 0 - 0.03 |
| | 14 | 0/5 | 0.0 | 0 |
| | 21 | 0/5 | 0.0 | 0 |
| | 28 | 0/5 | 0.0 | 0 |
| Methicillin | 0.25 | 5/5 | 84 | 3.7 - 47,794 |
| | 1 | 5/5 | 120 | 36.4 - 500,000 |
| | 3 | 4/5 | 3.2 | 0 - 144 |
| | 7 | 4/5 | 8.5 | 0 - 8,133 |
| | 14 | 2/5 | 0.0 | 0 - 0.3 |
| | 21 | 1/5 | 0.0 | 0 - 1,389 |
| | 28 | 1/5 | 0.0 | 0 - 1,153 |
| Controls | 0.25 | 10/10 | 333,510 | 84,821 - 1,133,333 |
| | 1 | 5/5 | 495,049 | 526 - 625,000 |
| | 3 | 4/4 | 2,011,546 | 8,607 - 19,555,556 |
| | 7† | – | – | – |

*Per mg kidney tissue.
†No survivors.

In contrast, positive kidney cultures were obtained with Wy-3206 on the 21st day and with methicillin and potassium penicillin G during the entire 28-day period (Fig. 1).

The results with Wy-3277 and methicillin at a dose level of 0.675 mg were somewhat comparable during the first two weeks (Fig. 2). Mice treated with methicillin were found to be negative for *S. aureus* on the third and fourth week of the study. On the other hand, in the group receiving Wy-3277 the percentage of infected kidneys increased during this period.

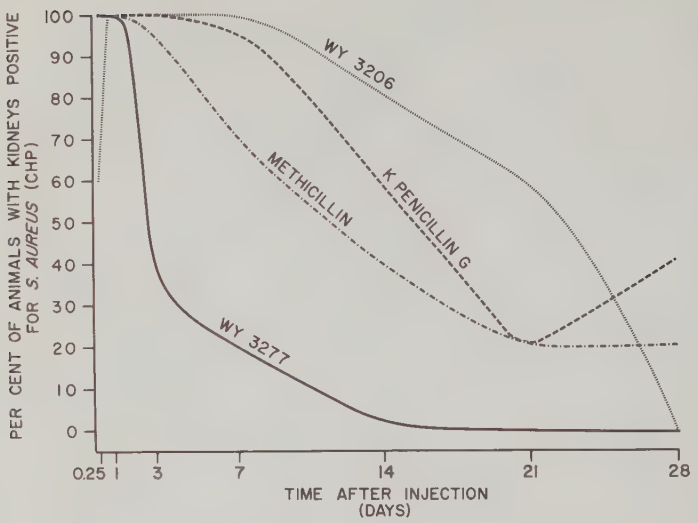


FIG. 1. *Fate of a penicillin G-resistant strain of Staphylococcus aureus (CHP) in kidneys of mice treated intramuscularly with a single dose (2.7 mg) of K penicillin G, Wy-3206, Wy-3277, and methicillin.*

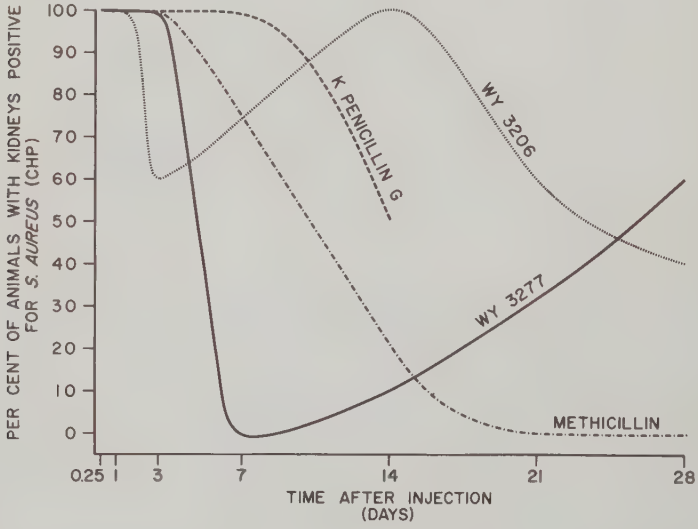


FIG. 2. *Fate of a penicillin G-resistant strain of Staphylococcus aureus (CHP) in kidneys of mice treated intramuscularly with a single dose (0.675 mg) of K penicillin G, Wy-3206, Wy-3277, and methicillin.*

TABLE 6. *Fate of Staphylococcus aureus strain CHP in kidneys of mice treated intramuscularly with a single dose (0.675 mg) of potassium penicillin G, Wy-3206, Wy-3277, and methicillin*

| Agent | Days after infection | No. animals positive (total) | Median viable count* | Range of counts* |
|---|---|---|---|---|
| Potassium penicillin G | 0.25 | 5/5 | 3,438 | 1,149 - 297,046 |
| | 1 | 5/5 | 388,266 | 3,451 - 432,900 |
| | 3 | 5/5 | 3,672 | 8.5 - 158,163 |
| | 7 | 5/5 | 814 | 7.9 - 617,162 |
| | 14 | 2/4 | 204 | 0 - 814 |
| | 21† | – | – | – |
| | 28† | – | – | – |
| Wy-3206 | 0.25 | 5/5 | 246 | 159 - 403 |
| | 1 | 5/5 | 197 | 14.5 - 26,341 |
| | 3 | 3/5 | 0.35 | 0 - 8,849 |
| | 7 | 4/5 | 0.32 | 0 - 394,350 |
| | 14 | 5/5 | 18.7 | 12 - 1,135,000 |
| | 21 | 3/5 | 0.43 | 0 - 0.73 |
| | 28 | 2/5 | 0.0 | 0 - 0.09 |
| Wy-3277 | 0.25 | 5/5 | 163 | 52 - 485 |
| | 1 | 5/5 | 78.5 | 40 - 296 |
| | 3 | 5/5 | 0.8 | 0.05 - 110 |
| | 7 | 0/5 | 0.0 | 0 |
| | 14 | 1/5 | 0.0 | 0 - 201,186 |
| | 21 | 1/5 | 0.0 | 0 - 0.02 |
| | 28 | 3/5 | 0.0 | 0 - 136 |
| Methicillin | 0.25 | 5/5 | 196 | 144 - 910 |
| | 1 | 5/5 | 243 | 24.6 - 24,891 |
| | 3 | 5/5 | 26 | 0.1 - 1023 |
| | 7 | 4/5 | 4.1 | 0 - 26,666 |
| | 14 | 1/5 | 0.0 | 0 - 0.05 |
| | 21 | 0/5 | 0.0 | 0 |
| | 28 | 0/5 | 0.0 | 0 |
| Controls | 0.25 | 10/10 | 333,510 | 84,821 - 1,133,333 |
| | 1 | 5/5 | 495,049 | 526 - 625,000 |
| | 3 | 4/4 | 2,011,546 | 8,607 - 19,565,556 |
| | 7† | – | – | – |

*Per mg kidney tissue.
†No survivors.

## Discussion

Since the original publication by Batchelor et al. (1959) on 6-aminopenicillanic acid, a wide variety of semisynthetic penicillins has been characterized. Clinical effectiveness of methicillin (Douthwaite and Trafford, 1960) against penicillin-resistant strains of *S. aureus* has led to the syntheses of a number of additional derivatives. Laboratory data on Wy-3206 and Wy-3277 have shown them to be at least as effective as methicillin in experimental infections in animals. The greater degree of activity of Wy-3206 and Wy-3277 against *D. pneumoniae* and *S. pyogenes* could be ascribed to structural differences.

Experimental data on the fate of *S. aureus* in the kidneys, after treatment with the four penicillins, show the effectiveness of Wy-3277 in this comparative study. Irrespective of the penicillins employed, the estimate of staphylococcal populations in the kidneys declined linearly within the first 7 days after a single intramuscular dose. The higher counts thereafter were usually attributable to a small proportion of the animals surviving. Biological variation apparently plays an important role as to whether a given animal may be rendered free from a persistent infection after treatment with the penicillins.

## Acknowledgment

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## Literature Cited

Batchelor, F. R., F. P. Doyle, J. H. C. Nayler, and G. N. Rolinson. 1959. Synthesis of penicillin: 6-aminopenicillanic acid in penicillin fermentations. Nature **183**:257-258.

Douthwaite, A. H., and J. A. P. Trafford. 1960. A new synthetic penicillin. Brit. Med. J. **2**: 687-690.

Reed, L. J., and H. Muench. 1938. A simple method of estimating fifty percent endpoints. Am. J. Hyg. **27**:493-497.

Rosenman, S. B., and G. H. Warren. 1962. In vitro evaluation of semisynthetic penicillins Wy-3206 and Wy-3277. Antimicrobial Agents and Chemotherapy - 1961, p. 611-619.

# ACTIVITY OF THE SODIUM SALT OF 6-(2-ETHOXY-1-NAPTHAMIDO-)PENICILLANIC ACID AGAINST STAPHYLOCOCCI IN VITRO

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A number of alterations in the structure of penicillin G have resulted in changes in antimicrobial activity and pharmacology. Methicillin is more active than penicillin G against large inocula of penicillinase-producing staphylococci, but less active against strains which do not produce penicillinase (White and Varga, 1961; Wallmark and Finland, 1961). Phenethicillin and α-phenoxypropyl penicillin are absorbed more completely after oral administration than penicillin G or penicillin V (Williams, Morrison, and Stevens, 1961; Morigi, Wheatley, and Albright, 1960), but methicillin is absorbed very poorly after oral administration (Cronk and Morigi, 1961).

A new penicillin, 6-(2-ethoxy-1-naphthamido)penicillanic acid (Wy-3277), has been prepared by Wyeth Laboratories (Radnor, Pa.). This compound inhibited the growth of large inocula of penicillinase-producing staphylococci at lower concentrations than did penicillin G, phenethicillin, or methicillin. Although Wy-3277 was less active on a weight basis than phenethicillin or penicillin G against staphylococci which do not produce penicillinase, it was more active than methicillin against these bacteria.

## Materials and Methods

Coagulase-positive staphylococci were isolated from the lesions of patients at Louisville General Hospital from January to May 1961. The bacteriostatic and bactericidal activity of four penicillins against the 50 strains were determined by tube-dilution methods (Petersdorf et al., 1960). The activity of penicillinase from the staphylococcal strains was determined by a modified Gots method (Haight and Finland, 1952), using each of the penicillins as a substrate.

All staphylococci were phage typed with bacteriophages propagated in heart infusion broth with added calcium (White, Foster, and Knight, 1959). Each strain was tested with dilute phages; those not reacting were retested with 1000-fold higher concentration.

The susceptibility of the staphylococci to tetracycline, erythromycin, chloramphenicol, kanamycin, and oleandomycin was determined by plate-dilution methods (Jackson and Finland, 1951). Staphylococci were considered resistant to a drug if they were not inhibited by 10 $\mu$g per ml.

## Results

Thirty of the 50 staphylococcal strains produced a penicillinase active against penicillin G after 24 hr incubation. The penicillinase producers reacted with phages 80 or 81 or both much more frequently than strains which did not produce penicillinase (Table 1).

TABLE 1. *Susceptibility of 50 staphylococci to staphylococcal bacteriophages*

| Phage type | Penicillinase producers (30 strains) | Nonproducers of penicillinase (20 strains) |
|---|---|---|
| | % | % |
| Group I | 0 | 5 |
| Group II | 10 | 15 |
| Group III | 10 | 5 |
| 80/81 | 50 | 15 |
| Nontypeable | 30 | 60 |

A large number of penicillinase-producing staphylococci were also resistant to tetracycline, erythromycin, and chloramphenicol; 10% were resistant to kanamycin, but none of the strains was resistant to oleandomycin (Table 2). Except for two strains resistant to tetracycline, the staphylococci which did not produce penicillinase were susceptible to all five antibiotics.

TABLE 2. *Susceptibility of 50 staphylococcal strains to tetracycline, erythromycin, chloramphenicol, kanamycin, and oleandomycin*

| Antibiotic | Strains resistant to antibiotic | |
|---|---|---|
| | Penicillinase producers (30 strains) | Nonproducers (20 strains) |
| | % | % |
| Tetracycline | 63 | 10 |
| Erythromycin | 40 | 0 |
| Chloramphenicol | 33 | 0 |
| Kanamycin | 10 | 0 |
| Oleandomycin | 0 | 0 |

Phenethicillin was destroyed by the 30 penicillinase producers in a manner similar to the activity against penicillin G. Some destruction of Wy-3277 could be demonstrated by 13 of the strains, but only after an additional 48 hr of incubation; at this time, slight destruction of methicillin had occurred with seven strains.

No destruction of phenethicillin, Wy-3277, or methicillin could be demonstrated when the 20 strains which did not destroy penicillin G were tested.

Against light and heavy inocula of staphylococci which did not produce penicillinase, penicillin G and phenethicillin were more active bacteriostatic and bactericidal agents than either Wy-3277 or methicillin (Tables 3, 4, and 5). However, Wy-3277 was more active against these strains than methicillin.

TABLE 3. *Bacteriostatic activity of four penicillins against 50 recently isolated staphylococci*

$10^{-4}$ Dilution of staphylococci

| Antibiotic | Cumulative per cent susceptible $\mu$g/ml | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 0.05 | 0.1 | 0.2 | 0.4 | 0.8 | 1.6 | 3.1 | 6.2 | 12.5 | 25 | 50 | 100 |
| | *Penicillinase producers (30 strains)* | | | | | | | | | | | |
| Wyeth 3277 | | 0 | 10 | 43 | 100 | | | | | | | |
| G | | | 10 | 23 | 40 | 43 | 43 | 47 | 57 | 80 | 97 | 100 |
| Phenethicillin | 0 | 10 | 17 | 33 | 43 | 50 | 63 | 77 | 77 | 90 | 100 | |
| Methicillin | | | | 0 | 3 | 10 | 73 | 100 | | | | |
| | *Nonpenicillinase producers (20 strains)* | | | | | | | | | | | |
| Wyeth 3277 | | 0 | 20 | 95 | 100 | | | | | | | |
| G | 55 | 100 | | | | | | | | | | |
| Phenethicillin | 16 | 100 | | | | | | | | | | |
| Methicillin | | | 0 | 5 | 30 | 95 | 100 | | | | | |
| | *All strains* | | | | | | | | | | | |
| Wyeth 3277 | | 0 | 14 | 64 | 100 | | | | | | | |
| G | 22 | 40 | 46 | 54 | 64 | 66 | 66 | 68 | 74 | 88 | 98 | 100 |
| Phenethicillin | 8 | 46 | 50 | 60 | 66 | 70 | 78 | 86 | 86 | 94 | 100 | |
| Methicillin | | | | 0 | 4 | 18 | 82 | 100 | | | | |

Wy-3277 inhibited, at lower concentrations than penicillin G or phenethicillin, the growth of most strains which produced penicillinase when light inocula were tested (Table 3, Fig. 1), and inhibited all strains at lower concentrations when heavier inocula were tested (Table 4, Fig. 2). Against both heavy and light inocula, Wy-3277 was more active than methicillin.

The bacteriostatic activity of the four penicillins was altered with increasing numbers of penicillinase-producing staphylococci. When 1:10,000 dilutions of overnight cultures were tested, all strains were inhibited by 100 $\mu$g per ml of penicillin G and by 50 $\mu$g per ml

of phenethicillin. When a 100-fold higher concentration of penicillinase-producing staphylococci was tested, only 20% of the strains were inhibited by 100 $\mu$g per ml of penicillin G and only 30% by 100 $\mu$g per ml of phenethicillin.

TABLE 4. *Bacteriostatic activity of four penicillins against 50 recently isolated staphylococci*

$10^{-2}$ Dilution of staphylococci

| Antibiotic | Cumulative per cent susceptible $\mu$g/ml | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | .05 | .1 | .2 | .4 | .8 | 1.6 | 3.1 | 6.2 | 12.5 | 25 | 50 | 100 |
| | *Penicillinase producers (30 strains)* | | | | | | | | | | | |
| Wyeth 3277 | | 0 | 7 | 40 | 80 | 93 | 100 | | | | | |
| G | | | | | | | | 0 | 3 | 3 | 10 | 20 |
| Phenethicillin | | | | | | | 0 | 7 | 10 | 23 | 27 | 30 |
| Methicillin | | | | | | 0 | 63 | 100 | | | | |
| | *Nonpenicillinase producers (20 strains)* | | | | | | | | | | | |
| Wyeth 3277 | | 0 | 30 | 75 | 100 | | | | | | | |
| G | 60 | 90 | 100 | | | | | | | | | |
| Phenethicillin | 35 | 90 | 100 | | | | | | | | | |
| Methicillin | | | | 0 | 5 | 30 | 95 | 100 | | | | |
| | *All strains* | | | | | | | | | | | |
| Wyeth 3277 | | 0 | 16 | 54 | 88 | 98 | 100 | | | | | |
| G | 24 | 36 | 40 | 40 | 40 | 40 | 40 | 40 | 42 | 42 | 46 | 52 |
| Phenethicillin | 14 | 36 | 40 | 40 | 40 | 40 | 40 | 44 | 46 | 54 | 56 | 58 |
| Methicillin | | | | 0 | 2 | 12 | 76 | 100 | | | | |

TABLE 5. *Bactericidal activity of four penicillins*

$10^{-4}$ Inoculum of staphylococci

| Antibiotic | Cumulative per cent susceptible $\mu$g/ml | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | .05 | .1 | .2 | .4 | .8 | 1.6 | 3.12 | 6.25 | 12.5 | 25 | 50 | 100 |
| | *Penicillinase producers (30 strains)* | | | | | | | | | | | |
| Wyeth 3277 | | | 0 | 10 | 43 | 70 | 93 | 100 | | | | |
| G | | | 0 | 7 | 13 | 13 | 27 | 30 | 37 | 43 | 53 | 63 |
| Phenethicillin | | | 0 | 7 | 10 | 17 | 23 | 33 | 37 | 50 | 50 | 57 |
| Methicillin | | | | | 0 | 7 | 33 | 87 | 97 | 97 | 100 | |
| | *Nonpenicillinase producers (20 strains)* | | | | | | | | | | | |
| Wyeth 3277 | | 0 | 10 | 40 | 90 | 100 | | | | | | |
| G | 25 | 85 | 95 | 100 | | | | | | | | |
| Phenethicillin | 15 | 55 | 90 | 100 | | | | | | | | |
| Methicillin | | 0 | 0 | 5 | 5 | 15 | 40 | 90 | 95 | 95 | 100 | |
| | *All strains* | | | | | | | | | | | |
| Wyeth 3277 | | 0 | 4 | 22 | 62 | 82 | 96 | 100 | | | | |
| G | 10 | 34 | 38 | 44 | 48 | 48 | 56 | 58 | 62 | 66 | 72 | 78 |
| Phenethicillin | 6 | 22 | 36 | 44 | 46 | 50 | 54 | 60 | 62 | 70 | 70 | 74 |
| Methicillin | | | 0 | 2 | 2 | 10 | 36 | 88 | 96 | 96 | 100 | |

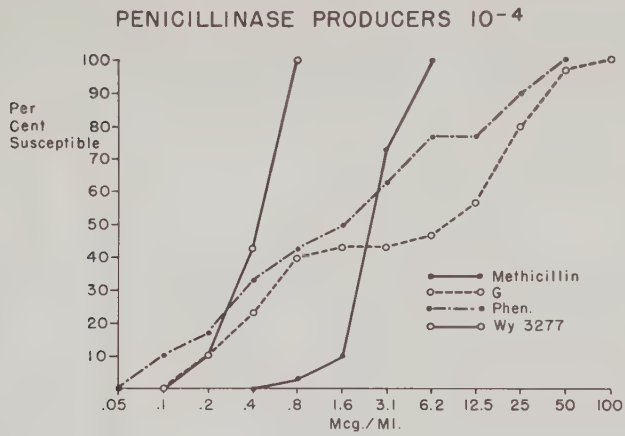


FIG. 1. *Bacteriostatic activity of four penicillins against 30 strains of penicillinase-producing staphylococci. Final concentration of staphylococci: $10^{-4}$ dilution of overnight cultures.*

The concentration of methicillin and Wy-3277 required to inhibit growth of penicillinase-producing staphylococci were altered to a lesser degree with increasing inocula. All strains were inhibited by 0.8 $\mu$g per ml of Wy-3277 and by 6.2 $\mu$g per ml of methicillin when light inocula of staphylococci were tested, and when heavy inocula were tested all strains were inhibited by 3.12 $\mu$g per ml of Wy-3277 and by 6.2 $\mu$g per ml of methicillin.

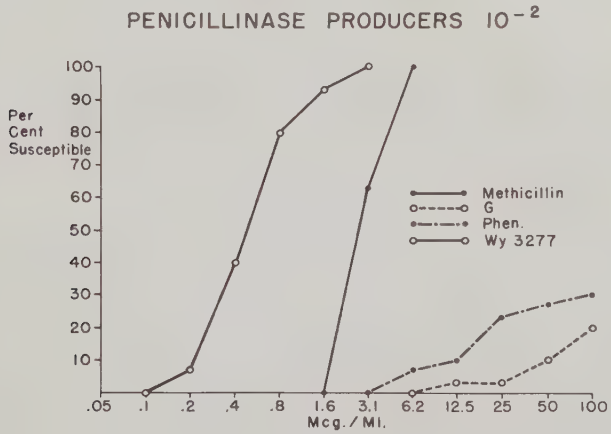


FIG. 2. *Bacteriostatic activity of four penicillins against 30 strains of penicillinase-producing staphylococci. Final concentration of staphylococci: $10^{-2}$ dilution of overnight cultures.*

Undiluted overnight broth cultures of an additional 200 staphylococcal strains from nasal carriers were tested by plate dilution methods. Although 33% of these strains were resistant to 50 μg per ml of penicillin G, all were susceptible to 1 μg per ml or less of Wy-3277.

## Discussion

An ideal antistaphylococcal agent against penicillinase-producing staphylococci would be as active on a weight basis as is penicillin G against staphylococci which do not produce penicillinase. In addition, it would be bactericidal, effective against large numbers of staphylococci, well absorbed from a variety of sites, and nontoxic.

The in vitro data presented here suggest that Wy-3277 approaches but does not attain these theoretical objectives. Wy-3277 was effective against large numbers of penicillinase-producing staphylococci and was bactericidal. Although not as active against penicillinase-producing staphylococci as penicillin G was against staphylococci which did not produce penicillinase, Wy-3277 was effective in lower concentrations against both of these types of bacteria than was methicillin.

These initial promising results will have to be considered together with toxicity data, absorption of the compound, the rate at which resistance might develop, and finally, clinical efficacy, before the complete role of Wy-3277 in the treatment of staphylococcal infections can be determined.

## Literature Cited

Cronk, G. A., and E. M. E. Morigi. 1961. Absorption, excretion and distribution of dimethoxyphenyl penicillin, p. 58-71. *In* P. A. Bunn [ed. ], A symposium on the new dimethoxyphenyl penicillin. State University of New York, Upstate Medical Center, Syracuse.

Haight, T. H., and M. Finland. 1952. Modified Gots test for penicillinase production. Am. J. Clin. Pathol. 22:806-808.

Jackson, G. G., and M. Finland. 1951. Comparison of methods for determing sensitivity of bacteria to antibiotics. A. M. A. Arch. Internal Med. 88:446-460.

Morigi, E. M. E., W. B. Wheatley, and H. Albright. 1960. Clinical and laboratory studies with potassium penicillin-152 (potassium (α-phenoxyethyl)-penicillin). Antibiotics Ann. 1959-60, p.127-132.

Petersdorf, R. G., M. C. Rose, H. R. Minchew, W. R. Keene, and I. L. Bennett, Jr. 1960. The sensitivity of hemolytic staphylococci to a series of antibiotics. II. A three year progress report. A. M. A. Arch. Internal Med. 105:398-412.

Wallmark, G., and M. Finland. 1961. Comparative activity of various penicillins against penicillinase-producing and non-penicillinase-producing staphylococci. Proc. Soc. Exptl. Biol. Med. 106:78-85.

White, A., F. Foster, and V. Knight. 1959. Propagation of staphylococcal phages in liquid medium. Antibiotics & Chemotherapy 9:81-86.

White, A., and D. T. Varga. 1961. Antistaphylococcal activity of dimethoxyphenyl penicillin, p. 146-159. *In* P. A. Bunn [ed. ], A symposium on the new dimethoxyphenyl penicillin. State University of New York, Upstate Medical Center, Syracuse.

Williams, G. M., J. K. Morrison, and K. S. Stevens. 1961. A new synthetic penicillin, PA 248. Lancet 1:847-850.

# BACTERIOLOGICAL COMPARISON OF L- AND D-PHENETHICILLIN

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After the discovery that the penicillin nucleus, 6-aminopenicillanic acid, could be obtained by a variety of technics, the ready synthesis of new and unique penicillins became possible. The first of such penicillins to be marketed commercially was the racemic mixture DL-phenoxyethyl penicillin (generic name, phenethicillin). As available commercially and as defined in the Federal Register, this mixture contains at least 55 to 75% of the L isomer and 25 to 45% of the D isomer. It would appear that biological activity of the racemic mixture is dependent upon the proportion of and the individual activity of each component isomer. Therefore, an increase in content of the isomer with greatest biological activity with a consequent reduction in content of the least active isomer might result in a phenethicillin with greater biological activity. Comparative in vitro and in vivo activities of phenethicillin, L-phenethicillin, and D-phenethicillin are presented in the present study.

## Materials and Methods

Samples of phenethicillin, L-phenethicillin, and D-phenethicillin were supplied to this laboratory by members of the Chemical Research Staff of Chas. Pfizer and Co., Inc. Phenethicillin, as the racemic mixture, consisted of approximately 66% L isomer and 34% D isomer. Samples designated as L isomer contained at least 95% L-phenethicillin and less than 5% D-phenethicillin. The converse was also true, i.e., the D isomer contained at least 95% D-phenethicillin and less than 5% L-phenethicillin.

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## Results

The minimal inhibitory concentrations (MIC), i.e., the least amount of antibiotic that prevented visible growth in brain heart infusion broth (Difco) after 18 to 20 hr incubation at 37 C, of phenethicillin, D-phenethicillin, and L-phenethicillin against eight pathogenic microorganisms are presented in Table 1. In each instance, the inoculum was 0.5 ml × $10^{-3}$ dilution of an 18-hr daily culture. Dilutions for the MIC test utilized an arithmetic progression technic in which the intervals between tubes varied, for example, as follows: 0.1, 0.2, 0.3, etc., μg/ml (Tompsett, Shultz, and McDermott, 1947). The standard deviation calculated and presented was based on N equals 3 or 5. In every instance, L-phenethicillin was approximately 2 to 4 times more active than the D isomer. However, within the limits of the standard deviation, phenethicillin and L-phenethicillin had the same MIC's against this group of test organisms.

TABLE 1. *In vitro activity of phenethicillin, L-phenethicillin, and D-phenethicillin against representative gram-positive bacteria*

| Organism | MIC* (μg/ml) | | |
|---|---|---|---|
| | Phenethicillin | D-Phenethicillin | L-Phenethicillin |
| *Staphylococcus* 5 | 0.073 ± 0.004 | 0.13 ± 0.036 | 0.073 ± 0.004 |
| *Staphylococcus* 209 P | 0.073 ± 0.004 | 0.166 + 0.046 | 0.066 ± 0.005 |
| *Streptococcus* 8668 | 0.016 ± 0.004 | 0.026 ± 0.004 | 0.016 ± 0.004 |
| *Streptococcus* 98 | 0.026 ± 0.004 | 0.036 ± 0.004 | 0.026 ± 0.004 |
| *S. faecalis* | 0.200† | 0.700 ± 0.024 | 0.23 ± 0.046 |
| *D. pneumoniae* | 0.200† | 0.66 ± 0.046 | 0.16 ± 0.046 |

*MIC = minimal inhibitory concentration (least amount of antibiotic preventing visible growth of test organism after 18 to 20 hr incubation at 37 C).

†Same value in all three tests.

Data obtained by the same arithmetic progression technic against a total of 11 *Diplococcus pneumoniae* and three *Streptococcus pyogenes* strains are presented in Table 2. These strains were freshly isolated, penicillin-susceptible, clinical cultures from two different local hospitals. Within the calculated standard deviation limits, the MIC for L-phenethicillin is lower (more active) against six strains of diplococci than that for D-phenethicillin. L-Phenethicillin was more active than D-phenethicillin against two of the three streptococci studied. However, L-phenethicillin has greater activity than phenethicillin against only three of the *D. pneumoniae* strains and against only a single streptococcus. An arithmetic average of these MIC values for the *D. pneumoniae* strains is as follows: phenethicillin, 0.037; D-phenethicillin, 0.045; and L-phenethicillin, 0.020 μg/ml. Similarly, averages for the streptococcal strains show 0.0437 μg/ml for phenethicillin, 0.058 μg/ml for D-phenethicillin, and 0.0285

TABLE 2. *In vitro activity of phenethicillin, L-phenethicillin, and D-phenethicillin against freshly isolated, clinical Streptococcus and Diplococcus strains*

| Culture | MIC* ($\mu$g/ml) | | |
|---|---|---|---|
| | Phenethicillin | D-Phenethicillin | L-Phenethicillin |
| Pneumococci | | | |
| 1 | 0.0344 (±0.022) | 0.0453 (±0.020) | 0.0187 (±0.01) |
| 2 | 0.0328 (±0.020) | 0.0437 (±0.020) | 0.0109 (±0.011) |
| 7 | 0.0359 (±0.020) | 0.0421 (±0.020) | 0.0117 (±0.004) |
| 8 | 0.0351 (±0.024) | 0.0406 (±0.020) | 0.0164 (±0.008) |
| 10 | 0.0359 (±0.025) | 0.0656 (±0.028) | 0.0468 (±0.015) |
| 11 | 0.0375 (±0.022) | 0.0468 (±0.039) | 0.0270 (±0.026) |
| 12 | 0.0344 (±0.023) | 0.0391 (±0.021) | 0.0242 (±0.021) |
| C | 0.0364 (±0.019) | 0.0574 (±0.028) | 0.0156 (±0)† |
| B | 0.0391 (±0.029) | 0.0449 (±0.026 | 0.0167 (±0.009) |
| D | 0.0521 (±0.010 | 0.0312 (±0)† | 0.0156 (±0)† |
| Streptococci | | | |
| 15 | 0.0437 (±0.016) | 0.0437 (±0.016) | 0.0293 (±0.007) |
| 16 | 0.0437 (±0.016) | 0.0625 (±0.033) | 0.0249 (±0.007) |
| 14 | 0.0437 (±0.016) | 0.0437 (±0.016) | 0.0312 (±0)† |

*MIC = minimal inhibitory concentration (least amount of antibiotic preventing visible growth of test organism after 18 to 20 hr incubation at 37 C).

†±0 = same reading in all tests.

$\mu$g/ml for L-phenethicillin. Again, L-phenethicillin is about twice as active as is D-phenethicillin, and appears about 18% more active than phenethicillin.

A summary of MIC data collected from 54 freshly isolated, penicillin-susceptible, clinical *Staphylococcus aureus* cultures is presented in Table 3. These cultures were received from seven different hospitals representing four general geographical areas. The MIC was determined for each strain based on 2 to 3 separate experiments done on different days. The arithmetic progression technique of dilutions was used. An arithmetic average was calculated for each culture. These were then again averaged to give the figure

TABLE 3. *MIC data from 54 freshly isolated penicillin-susceptible cultures of Staphylococcus aureus*

| Hospital source | No. of cultures | Average MIC ($\mu$g/ml) | | |
|---|---|---|---|---|
| | | Phenethicillin | D-Phenethicillin | L-Phenethicillin |
| Long Island A | 11 | 0.045 | 0.13 | 0.038 |
| Philadelphia | 5 | 0.056 | 0.24 | 0.036 |
| Brooklyn A | 6 | 0.058 | 0.26 | 0.056 |
| Manhattan A | 8 | 0.127 | 0.24 | 0.111 |
| Brooklyn B | 2 | 0.045 | 0.15 | 0.045 |
| Long Island B | 10 | 0.080 | 0.206 | 0.070 |
| Manhattan B | 12 | 0.072 | 0.41 | 0.063 |
| Average of the 54 cultures | | 0.068 | 0.224 | 0.062 |

presented in the table for individual hospital cultures. Again, in every instance the average MIC for L-phenethicillin is lower (more active) than that for D-phenethicillin (Table 3). Although the differences are slight, the average MIC's as presented for cultures from five of the seven hospitals show L-phenethicillin to be lower than the corresponding value for phenethicillin. Over-all average MIC's of the 54 cultures show phenethicillin, 0.068; D-phenethicillin, 0.224; and L-phenethicillin, 0.062 μg/ml. L-Phenethicillin appears about three times more active than D-phenethicillin. Again, L-phenethicillin and phenethicillin have almost identical average MIC values.

As was true in broth dilution tests, L-phenethicillin appears more active than D-phenethicillin in the usual antibiotic-susceptibility test done by the disc method. L-Phenethicillin, phenethicillin, and D-phenethicillin were pipetted onto sterile filter paper discs to give concentrations of 2 and 10 μg. These were then placed on the surface of the seeded agar plates. After 18 hr of incubation at 37 C, zones of inhibition were measured (Table 4). It is obvious that larger zones were produced by L-phenethicillin than by D-phenethicillin. Again, differences between zones produced by phenethicillin and L-phenethicillin were not significant.

TABLE 4. *Zone sizes produced by phenethicillin, L-phenethicillin, and D-phenethicillin against representative gram-positive bacteria*

| Penicillin | Disc concentration | Zone sizes | | | |
|---|---|---|---|---|---|
| | | *S. aureus* | *S. pyogenes* | *S. faecalis* | *D. pneumoniae* |
| | μg | mm | mm | mm | mm |
| Phenethicillin | 2 | 33 | >35 | 23.6 | >35 |
| | 10 | >35 | >35 | 25.9 | >35 |
| D-Phenethicillin | 2 | 30.6 | >35 | 17.6 | 35 |
| | 10 | 31.6 | >35 | 20 | >35 |
| L-Phenethicillin | 2 | 32.4 | >35 | 22.5 | >35 |
| | 10 | 35 | >35 | 27.8 | >35 |

A comparison of the chemotherapeutic efficacy of phenethicillin, L-phenethicillin, and D-phenethicillin against an experimental *S. aureus* infection in mice is presented in Table 5. The infection was produced by the intraperitoneal injection of a standardized culture of *S. aureus* in 5% hog gastric mucin. Therapy was initiated 0.5 hr later by either the oral or subcutaneous routes. Single dosage was used. It is obvious that L-phenethicillin is significantly more active against this experimental infection in mice than is D-phenethicillin administered either orally or subcutaneously. L-Phenethicillin had $PD_{50}$ (that dose in mg/kg which will protect 50% of the animals) orally of 0.82 with 95% confidence limits of 1.02 to 0.66 mg/kg. The oral $PD_{50}$ of D-phenethicillin, however, was 1.65 with confidence limits of 2.06 to 1.37 mg/kg. When administered subcutaneously,

L-phenethicillin had a $PD_{50}$ of 0.62 mg/kg with confidence limits of 0.84 to 0.46; D-phenethicillin was 1.10 mg/kg with confidence limits of 1.40 to 0.94. It is noted that, therefore, the in vivo chemotherapeutic activity of L-phenethicillin is about twice that of D-phenethicillin. This is identical with in vitro indications. As was true with in vitro data, L-phenethicillin and phenethicillin are not significantly different in activity against this experimental staphyloccal infection.

TABLE 5. *Chemotherapeutic activity of phenethicillin, L-phenethicillin, and D-phenethicillin against experimental Staphylococcus aureus infection in mice*

| Penicillin | $PD_{50}$ Mg/kg (95% confidence limits) | |
|---|---|---|
| | Oral | Parenteral |
| Phenethicillin | 0.95 (1.16-0.78) | 0.70 (0.92-0.52) |
| D-Phenethicillin | 1.65 (2.06-1.37) | 1.10 (1.40-0.94) |
| L-Phenethicillin | 0.82 (1.02-0.66) | 0.62 (0.84-0.46) |

## Discussion

It has been claimed that the diastereoisomeric mixture comprising phenethicillin has greater activity than that of its most active component (Gourevitch, Hunt, and Lein, 1960). However, the data obtained in our current studies are not in consistent agreement with this. L-Phenethicillin is certainly two to four times more active in vitro than is D-phenethicillin. However, the data for L-phenethicillin and phenethicillin are equivocal. In several instances, L-phenethicillin had greater in vitro activity than phenethicillin; in other instances, the converse was true. Similar findings have been reported by others. McCarthy and Finland (1960), by a twofold dilution technique, showed L-phenethicillin and phenethicillin to have greater activity than D-phenethicillin. In addition, the MIC figure presented for L-phenethicillin was lower than that of phenethicillin; however, these differences ranged only from 0.006 to 0.02 μg/ml (see Table 1 of reference). Wallmark and Finland (1961) showed L-phenethicillin and phenethicillin to have equal in vitro activity against nonpenicillinase-producing staphylococci. Both were about four times more active than D-phenethicillin. McCarthy, Wallmark, and Finland (1961) in a later paper pointed out, "among the phenethicillins, the L isomer was the most active, the D isomer the least active and the activity of the DL form was intermediate, but more nearly approached that of the L form" as related to studies on gram-positive organisms.

In our chemotherapeutic studies against an experimental *S. aureus* infection in mice, L-phenethicillin and phenethicillin were clearly

superior in activity to D-phenethicillin. However, the differences between the L isomer and phenethicillin were not statistically significant. Gourevitch et al. (1959), reported $CD_{50}$ (curative dose) in mg/kg (as geometric means) against their experimental staphylococcal infection in mice, D-phenethicillin, 0.92; L isomer, 0.53; and phenethicillin, 0.32 mg/kg. However, as they mentioned, the differences between the L isomer and phenethicillin was not great enough to be significant statistically at the 0.05 probability level.

In another in vivo evaluation test, Cronk et al. (1960) reported that blood levels in man after oral administration of the L isomer and phenethicillin were about twice that obtained with similar administration of the D isomer. However, L-phenethicillin and phenethicillin gave almost identical results.

Because of the limits of error in various biological procedures, we can only generalize that the activity of phenethicillin (66% L isomer and 34% D isomer) usually reflected that which one might expect if each component was acting separately. D-Phenethicillin is obviously less active both in vitro and in in vivo than is the L isomer.

## Literature Cited

Cronk, G. A., D. E. Nauman, H. Albright, and W. B. Wheatley. 1960. Laboratory and clinical studies with potassium penicillin-152 [potassium (α-phenoxyethyl)-penicillin]. Antibiotics Ann. 1959-1960, p. 133-145.

Gourevitch, A., G. A. Hunt, and J. Lein. 1960. Microbiological studies on potassium penicillin-152 [potassium (α-phenoxyethyl)-penicillin]. Antibiotics Ann. 1959-1960, p. 111-118.

McCarthy, C. G., and M. Finland. 1960. Absorption and excretion of four penicillins: penicillin G. penicillin V, phenethicillin and phenylmercaptomethyl penicillin. New Engl. J. Med. 263: 315-326.

McCarthy, C. G., G. Wallmark, and M. Finland. 1961. *In vitro* activity of various penicillins. Am. J. Med. Sci. 241:145-159.

Tompsett, R., S. Shultz, and W. McDermott. 1947. The relation of protein binding to the pharmacology and antibacterial activity of penicillins X, G, dehydro F, and K. J. Bacteriol. 53: 581-595.

Wallmark, G., and M. Finland. 1961. Comparative activity of various penicillins against penicillinase-producing and nonpenicillinase-producing staphylococci. Proc. Soc. Exptl. Biol. Med. 106:78-85.

# EFFECT OF METHICILLIN ON THE DISAPPEARANCE OF RESISTANT *STAPHYLOCOCCUS AUREUS* FROM SOFT-TISSUE LESIONS

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The purpose of this study was to determine the rapidity of removal of staphylococci from soft tissue lesions of patients treated with methicillin (Douthwaite and Trafford, 1960) compared with local treatment alone. The methicillin group included 11 patients at Longview State Hospital who developed furuncles, decubiti, or postoperative infections. Ten control patients with comparable lesions received only conservative treatment.

## Materials and Methods

*Cultures.* Daily swabs were taken from the involved lesions for the first three days and thereafter at weekly or more frequent intervals, depending upon the condition of the patient. The swabs were streaked on blood agar and mannitol agar and incubated at 37 C. The number of colonies on the mannitol plate was estimated after 48 hr of incubation, and four colonies were picked and tested against penicillin G and methicillin by serial dilution tests and against other antibiotics by agar diffusion, using filter paper discs. These four colonies were also phage typed. A second swab from each was smeared for gram staining. Cultures from the right and left nostrils were taken separately from some of the patients early in their course and from six patients after their lesions had subsided.

*Blood levels.* Pairs of blood samples were drawn from each treated patient 2 and 5.5 hr after a dose of methicillin, usually on three successive days, for measurement of methicillin serum levels. The concentration in $\mu$g per ml was determined by a twofold serial dilution method in broth (Hamburger and Muething, 1951), employing *Bacillus subtilis* as the test organism. In addition, the inhibitory

activity of each serum against the *Staphylococcus* of the patient was tested in a twofold serial dilution test, employing a $10^{-2}$ inoculum of staphylococci. Since 0.5 ml of serum, or serum dilution, and 1.5 ml of inoculum comprised each test, activity in a dilution of less than 1:4 could not be detected. A swab from each clear tube was streaked on an agar plate as a crude measurement of bactericidal activity.

The methicillin-treated cases received 1 g intramuscularly every 6 hr and probenecid, 0.5 g orally every 6 hr. Hot compresses were used locally, and incision and drainage when and where indicated, in both methicillin and control patients.

## Results

The lesions of all patients improved, although improvement was noticeably slower in the control series than in the methicillin-treated patients. In the treated patients, the rapid drop-off in the number of staphylococci on the mannitol plate averaged 5 days, with a range of 1 to 14 days. In the control series the drop-off in staphylococci on the mannitol plates was observed only after 5 to 30 days, with an average of 13.1. Statistical analysis showed that the probability of this difference occurring by chance lay between 1 and 5%. Completely negative cultures were obtained in the treated group on an average of 8.7 days, with a range of 1 to 27, whereas in the control group, completely negative cultures were secured only after an average of 23.7 days, with a range of 18 to 33. The likelihood of this difference occurring by chance was 0.1%. Not included in these averages are one fatal case where no drop-off at all had occurred after 19 days of methicillin treatment, and three control cases where cultures were still positive at the termination of 16, 28, and 48 days of observation.

Tables 1 and 2 present data indicating that the clinical types of lesions and the phage types of the infecting staphylococci were similar in the two series.

The methicillin cases are listed in Table 1. More than half the infecting staphylococci were lysed by phages 80 and 81. The longest period before drop-off occurred was 14 days and sterile cultures were obtained in the majority of cases in less than a week. The duration of methicillin treatment ranged from 8 to 36 days, with an average of 20.5 days. Treatment was continued for an average of 15.8 days after rapid drop-off in staphylococci was noted and 12.1 days after cultures became negative.

The conservatively treated patients are listed in Table 2. It is evident that the lesions in the conservatively treated group were similar to those of the methicillin-treated patients. Here too, the infecting staphylococci from half the patients were lysed by phage 80

TABLE 1. *Rate of disappearance of Staphylococcus aureus from cultures of soft tissue lesions in methicillin-treated cases*

| Case | Location of lesion | Phage type | Rapid drop-off in no. of staphylococci | Negative culture |
|---|---|---|---|---|
| | | | *days* | *days* |
| 1 | Right elbow | 80/81 | 5 | 16 |
| 2 | Left elbow | 6/42E/47/54 | 12 | 12 |
| 3 | Right hip incision | 80/81 | 6 | 27 |
| 4 | Left inguinal incision | 7/47/53/54/77 | 1 | 1 |
| 5 | Left hip incision | 80/81 | 3 | 3 |
| 6 | Right thigh | 80/81 | 2 | 6 |
| 7 | Perineum | UNT* | 1 | 2 |
| 8 | Left thigh | 80/81 | 2 | 2 |
| 9 | Right radical mastectomy incision | 52/80 | 4 | 4 |
| 10 | Left scapula | 80/81 | 14 | 14 |
| 11 | Perineum | UNT | – | – |

*Symbols used: (UNT) untypable; (–) indicates that no drop-off occurred during the observation period (12 days in case 11).

TABLE 2. *Rate of disappearance of Staphylococcus aureus from cultures of soft-tissue lesions in patients with conservative treatment (no chemotherapy)*

| Case | Location of lesion | Phage type | Rapid drop-off in no. of staphylococci | Negative culture |
|---|---|---|---|---|
| | | | *days* | *days* |
| 1 | Right great toe | 80; UNT* | – | – |
| 2 | Abdominal hysterectomy incision | UNT | 5 | 30 |
| 3 | Left thigh | 80/81; 7/47/53/54/44 | 9 | 19 |
| 4 | Left heel | 80/81; UNT | 12 | – |
| 5 | Right lateral chest | UNT | 18 | 18 |
| 6 | Right inguinal incision | UNT | 33 | 33 |
| 7 | Right thumb; | UNT; 6/42E; 3A | 8 | 26 |
| | Right great toe | 187; UNT; 6/42E | 20 | 20 |
| 8 | Lower lip | UNT; 6/42E/47/54 | 8 | 26 |
| 9 | Right thigh | 80/81 | 5 | 18 |
| 10 | Lower extremities | 80/81 | – | – |

*Symbols used: (UNT) untypable; (–) indicates that no drop-off (or negative culture) occurred and cultures remained positive during the observation period (16 days in case 1; 28 days in case 4; and 48 days in case 10).

or 80 and 81. In contrast with the treated patients, where rapid drop-off usually occurred in less than a week and sometimes in 1 or 2 days, the sharp diminution in numbers of staphylococci on the mannitol plates usually required 8 days or longer. In cases 1 and 10, no drop-off was observed at the termination of the observation period, namely 16 and 48 days. Negative cultures were never obtained before 18 days and cultures often remained positive for 3 or 4 weeks.

The direct gram-stained smears did not reflect the results from the cultures, except that when staphylococci were found on the smears there were usually too many colonies to count on the agar plates. However, the cultures were often florid when the smears were negative or showed only rare cocci.

In three of the treated cases, relapse occurred after cessation of treatment. In one case methicillin was discontinued after 2 weeks of negative cultures, but 7 days later a heavy growth of staphylococci again appeared. In a second case, where methicillin was discontinued after 19 days of negative cultures, heavy growth occurred 4 days after methicillin was stopped. In one of these cases a metal prosthesis was present in the lesion, and in the second, large areas of necrotic tissue in multiple decubiti.

Using an average of 10 cases after an intramuscular dose, a high level of methicillin (16 $\mu$g/ml) was present in the serum after 2 hr, and an adequate level (4 $\mu$g/ml) was present after 5.5 hr. The uniformity of these levels, measured with *B. subtilis* as a test organism, is indicated by the fact that 85% of 2-hr levels and 90% of the 5.5-hr levels were within one twofold dilution tube of the concentration nearest these averages.

TABLE 3. *Activity of patients' sera against their own staphylococci*

| Serum dilutions | Sera inhibiting staphylococci | |
|---|---|---|
| | 2 hr after 1 g methicillin im | 5.5 hr after 1 g methicillin im |
| | % | % |
| 1:32 or more | 9.5 | 0 |
| 1:16 or more | 47.6 | 9.1 |
| 1:8 or more | 71.4 | 18.2 |
| 1:4 or more | 81.0 | 31.8 |
| <1:4 | 19.0 | 68.2 |

The capacity of the patients' sera to inhibit their own staphylococci in a serial dilution test is shown in Table 3. At the end of 2 hr there was good antistaphylococcal activity, since 81% of the specimens inhibited their own staphylococci in an overnight test, when the serum was diluted 1:4 or more. By the end of 5.5 hr, however, only 32% of the sera retained this capacity. Cultures of the clear tubes

in these tests practically always yielded varying numbers of staphylococci, indicating little or no bactericidal activity. It is of interest, and may or may not be of importance, that the only patients whose serum displayed no antistaphylococcal activity against their own organisms, despite the presence of adequate amounts of methicillin as judged by the tests with *B. subtilis*, were those who relapsed after the cessation of treatment.

Antibiotic sensitivity studies showed that, in the methicillin-treated cases, every strain required more than 25 μg of penicillin G/ml for growth inhibition. Of the 11 infecting strains, seven required 3.12 μg of methicillin, and four required 1.56 μg. Agar-diffusion disc tests showed most of the strains to be resistant to streptomycin, tetracycline, and sulfonamides, but, in general, sensitive to erythromycin, chloramphenicol, novobiocin, and vancomycin. The sensitivity patterns of the staphylococci from the control cases were similar, except that 60% of the strains were sensitive to penicillin G and 80% required only 1.56 μg instead of 3.12 μg of methicillin.

Cultures of the right and left nostrils were made 27 to 51 days after the cessation of methicillin treatment in five patients and during the third week of treatment in one patient. The cultures showed that two patients still harbored large numbers of staphylococci of the same type as was present in their soft tissue lesion; three harbored staphylococci of a different phage type from that in the lesion; and one had a negative nose culture.

## Discussion

It is concluded that the addition of methicillin to local treatment of soft tissue staphylococcal lesions brings about a more rapid healing of the lesions and a more rapid diminution and final disappearance of the infecting staphylococci. Methicillin treatment, however, can not be depended upon to eradicate the nasal carrier state. Since open lesions are recognized as a major source of staphylococci in hospital wards, a measure which diminishes the numbers of these organisms shed into the environment is a worthwhile step in the prevention of spread of infection, even in the face of occasional persisting nasal carrier states.

## Acknowledgment

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## Literature Cited

Douthwaite, A. H., and J. A. P. Trafford. 1960. A new synthetic penicillin. Brit. Med. J. 2:687-690.

Hamburger, M., and D. Muething. 1951. The in vitro sensitivity to eight antibiotics of streptococci and staphylococci recovered from cases of bacterial endocarditis. J. Lab. Clin. Med. 37: 60-63.

# A NEW SEMISYNTHETIC PENICILLIN. SK&F 12141[1]

## I. Microbiological Studies

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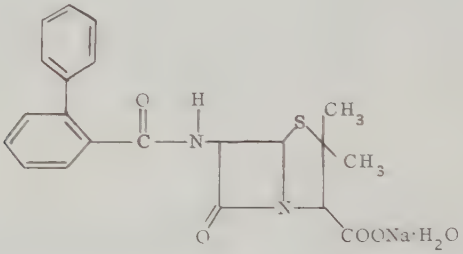
SK&F 12141, 2-biphenylylpenicillin, is a synthetic penicillin obtained by the acylation of 6-aminopenicillanic acid. It is isolated and tested as its monohydrate sodium salt.




SK&F 12141 shows activity against strains of penicillin-resistant staphylococci and is orally absorbed. In studying the biological properties of this compound it was compared with 2,6-dimethoxyphenylpenicillin (DMP) and phenoxymethylpenicillin (penicillin V). All three compounds were studied simultaneously in the same test systems.

### Materials and Methods

*Antibacterial spectrum.* Minimal inhibitory concentrations were determined against a variety of organisms by serial twofold dilutions of the test compounds in appropriate media. End points were determined by macroscopic readings after 18 hr of incubation at 37 C.

*Activity against penicillin-resistant strains of staphylococci.* Recent clinical isolates of penicillin-resistant staphylococci were studied by serial tube dilutions in Trypticase soy broth. The inoculum

[1]Now given generic name ancillin.

consisted of $1 \times 10^6$ organisms per ml. After incubation for 18 hr at 37 C, end points were read.

*Resistance to staphylococcal penicillinase.* A heavy suspension (3 to 5 mg dry weight) of penicillinase-producing staphylococci was used as a source of penicillinase. The constant-volume Warburg respirometer was used to measure penicillinase susceptibility of the test compounds. The method used is that of Henry and Housewright (1947) and is based on the principle that penicillinase acts upon penicillins by splitting the $\beta$-lactam ring structure to produce a new free carboxyl group. The quantity of new free carboxylic acid produced is measured by determining the amount of carbon dioxide released from sodium bicarbonate in the solution.

*Effect of inoculum size.* Trypticase soy broth cultures (18-hr cultures) of penicillin-susceptible and penicillin-resistant strains of *Staphylococcus aureus* were diluted to obtain inocula varying between $3 \times 10^5$ and $3 \times 10^8$ organisms per ml. Minimal inhibitory concentrations were determined from serial twofold dilutions of the test compounds in Trypticase soy broth.

*Stability in gastric fluid.* The stability of the compounds in synthetic gastric fluid, pH 1.6 (USP), was determined by assay of the solutions after incubation at 37 C for periods of 30 and 90 min. The residual penicillin was determined by the standard agar-diffusion method utilizing *Bacillus subtilis* as the test organism (Grove and Randall, 1955).

*Protein binding.* Each test compound (100 $\mu$g) was solubilized in 5 ml of pooled human serum. The samples were placed in cellulose dialysis bags and suspended in 20 ml of 0.85% sodium chloride solution. Dialysis was carried out for 48 hr at 4 C. The serum-containing residues and dialyzates were removed, volumes measured, and the concentration of the antibiotic in all of the solutions was determined by assay. The percentage binding was determined from the ratios of the antibiotic in the residue and dialyzate.

*Induction of staphylococcal penicillinase.* Induction of penicillinase by the test compounds was studied by the method of Geronimus and Cohen (1957) and Moat, Ceci, and Bondi (1961). The optimal concentration of each compound needed to produce induction of penicillinase within a 30-min exposure time was determined.

*Mouse infection-protection tests.* Pneumococcal infections were produced by intraperitoneal injection of 0.5 ml of a $10^{-6}$ dilution of an overnight broth culture of *Diplococcus pneumoniae,* type I. Compounds were administered by oral intubation in 0.5-ml volumes 1 and 18 hr after infection. The animals were observed for 1 week after infection.

Streptococcal infections were produced by intraperitoneal injection of a $10^{-4}$ dilution of an overnight broth culture of *Streptococcus pyogenes* C203. Test compounds were administered orally, immediately after infection and again 24 hr later. The animals were observed for 1 week after infection.

Staphylococcal infections were produced by injection of the tail vein with 0.3 ml ($2 \times 10^8$ organisms) of a washed suspension of *S. aureus* Finland 400 (penicillin resistant). Test compounds were administered by oral intubation 3 hr after infection and again 24 and 48 hr later. The animals were observed for the following 10 days.

## Results

*Antibacterial spectrum.* SK&F 12141 shows, in general, the same spectrum of activity as do DMP and penicillin V. With few exceptions, SK&F 12141 was more active than DMP and less active than penicillin V in inhibiting penicillin-susceptible organisms in vitro (Table 1).

TABLE 1. *Spectrum of SK&F 12141, DMP, and penicillin V*

| Organism | Minimal inhibitory concentration (μg/ml) | | |
|---|---|---|---|
| | SK&F 12141 | DMP | Penicillin V |
| *Bordetella pertussis* | 1.5 | 0.75 | 1.5 |
| *Brucella abortus* | 250 | 250 | 62.5 |
| *Clostridium perfringens* | 0.09 | 0.18 | 0.37 |
| *Corynebacterium diphtheriae* | 1.5 | 3.1 | 0.045 |
| *Diplococcus pneumoniae* | 0.11 | 0.11 | 0.025 |
| *Escherichia coli* | 500 | 1,500 | 500 |
| *Neisseria catarrhalis* | 0.18 | 0.75 | 0.022 |
| *Neisseria gonorrhoeae* | 0.75 | 0.37 | 0.022 |
| *Pasteurella multocida* | 1.5 | 3.1 | 0.75 |
| *Proteus vulgarus* | >2,000 | >2,000 | 500 |
| *Pseudomonas aeruginosa* | 1,500 | 2,000 | >2,000 |
| *Salmonella typhosa* | 500 | 1,000 | 1,500 |
| *Staphylococcus aureus* 209 P | 0.22 | 0.9 | 0.04 |
| *Streptococcus faecalis* | 62 | 500 | 15 |
| *Streptococcus pyogenes* C-203 | 0.045 | 0.09 | 0.002 |
| *Streptococcus salivarius* | 31 | 125 | 1.8 |

*Activity against penicillin-resistant strains of staphylococci.* SK&F 12141 inhibits strains of staphylococci at levels of 0.45 to 0.9 μg/ml. These same strains are inhibited by DMP at levels of 1.8 to 3.7 μg/ml, and penicillin V inhibits only at 1,000 to 2,000 μg/ml (Table 2).

*Resistance to staphylococcal penicillinase.* SK&F 12141, like DMP, resists destruction by staphylococcal penicillinase. Penicillin V is completely destroyed within 20 min (Table 3).

*Effect of inoculum size.* Against penicillin-susceptible staphylococci, the minimal inhibitory concentrations of SK&F 12141, DMP, and penicillin V are not affected by inoculum size.

Against penicillin-resistant staphylococci the minimal inhibitory concentration of SK&F 12141 is increased approximately fourfold

by increasing the inoculum by a factor of one thousand. This change is of the same order as that of DMP under the same conditions. In contrast, penicillin V shows a sharp decrease in activity with each increase in inoculum (Table 4).

TABLE 2. *Minimal inhibitory concentrations of SK&F 12141, DMP, and penicillin V against resistant staphylococci*

| SK&F strain number | Phage type | Minimal inhibitory concentration (μg/ml) | | |
|---|---|---|---|---|
| | | SK&F 12141 | DMP | Penicillin V |
| 24390 | 54 | 0.45 | 1.8 | 1,000 |
| 25390 | 80/81 | 0.45 | 1.8 | 1,500 |
| 26390 | 75/77/47/53/54 | 0.45 | 1.8 | 1,000 |
| 30390 | 80/81 | 0.9 | 1.8 | 1,500 |
| 31390 | 80/81 | 0.45 | 1.8 | 1,000 |
| 38390 | 80/81 | 0.45 | 1.8 | 1,500 |
| 40390 | 80/81 | 0.9 | 3.7 | 1,500 |
| 41390 | 80/81 | 0.9 | 3.7 | 1,500 |
| 42390 | 80/81 | 0.9 | 1.8 | 2,000 |
| 43390 | 80/81 | 0.9 | 3.7 | 1,500 |
| 44390 | 80/81 | 0.9 | 3.7 | 1,500 |
| 39390 | 47/54/77 | 0.45 | 1.8 | 1,500 |
| 45390 | Not typed | 0.9 | 3.7 | 1,500 |
| 47390 | Not typed | 0.9 | 1.8 | 1,500 |
| 48390 | Not typed | 0.9 | 3.7 | 2,000 |
| 49390 | Not typed | 0.9 | 3.7 | 2,000 |
| 51390 | 80/81 | 0.9 | 3.7 | 2,000 |
| 52390 | 80/81 | 0.9 | 3.7 | 1,500 |

TABLE 3. *Destruction of SK&F 12141, DMP, and penicillin V by staphylococcal penicillinase*

| Test compound | Molarity | Liters of $CO_2$ evolved | |
|---|---|---|---|
| | | 10 Min | 20 Min |
| Penicillin V | 0.03 | 307 | 329 |
| DMP | 0.03 | 2 | 5 |
| SK&F 12141 | 0.03 | 10 | 13 |

*Stability in gastric fluid.* SK&F 12141 resists destruction by synthetic gastric fluid (pH 1.6) to the same extent as penicillin V in the same test system. At the end of the 90-min period of testing, 82% of SK&F 12141 and 80% of penicillin V were recovered. Less than 9% of DMP remained after 90 min.

*Protein binding.* After dialysis for 48 hr at 4 C, 75% of SK&F 12141 remained bound to serum protein. In the same test, 48 and 68.6% of DMP and penicillin V, respectively, remained bound. The results reported here on penicillin V agree well with those reported by Kunin (1961).

TABLE 4. *Effect of inoculum size on susceptible and resistant Staphylococcus aureus*

| *S. aureus* strain | Inoculum | | MIC of test compound (μg/ml) | | |
|---|---|---|---|---|---|
| | Dilution | No. of organisms/ml | SK&F 12141 | DMP | Penicillin V |
| Penicillin susceptible #20390 | $10^{-1}$ | $7 \times 10^7$ | 0.45 | 0.9 | 0.045 |
| | $10^{-2}$ | $7 \times 10^6$ | 0.45 | 0.9 | 0.045 |
| | $10^{-3}$ | $7 \times 10^5$ | 0.45 | 0.9 | 0.045 |
| | $10^{-4}$ | $7 \times 10^4$ | 0.45 | 0.9 | 0.045 |
| Penicillin resistant #24390 | $10^{-1}$ | $3 \times 10^8$ | 1.8 | 3.7 | >4,000 |
| | $10^{-2}$ | $3 \times 10^7$ | 1.8 | 1.8 | 1,000 |
| | $10^{-3}$ | $3 \times 10^6$ | 0.9 | 1.8 | 125 |
| | $10^{-4}$ | $3 \times 10^5$ | 0.45 | 0.9 | < 62.5 |

*Induction of staphylococcal penicillinase.* SK&F 12141 induces penicillinase at approximately the same concentration as does DMP. Penicillin G is a relatively poor penicillinase inducer (Table 5).

TABLE 5. *Induction of staphylococcal penicillinase**

| Inducer | Optimal concn for induction | Molarity | $CO_2$ per mg at 30 min |
|---|---|---|---|
| | *μg/ml* | $6 \times 10^{-3}$ M | *μliters* |
| Penicillin G | 627 | 1,780 | 324 |
| DMP | 0.8 | 1.78 | 1,703 |
| SK&F 12141 | 0.8 | 1.78 | 1,820 |

*Penicillin G, $6 \times 10^{-3}$ M, was used as substrate.

*Mouse infection-protection tests.* Oral administration of SK&F 12141 produces a protective effect in pneumococcal infections. This

TABLE 6. *Oral efficacy of SK&F 12141 in mice infected with Diplococcus pneumoniae**

| Test compound | Oral dose | Survival after infection | |
|---|---|---|---|
| | | 3 days | 7 days |
| | *mg/kg* | % | % |
| SK&F 12141 | 10 | 80 | 30 |
| SK&F 12141 | 20 | 100 | 60 |
| SK&F 12141 | 40 | 100 | 100 |
| SK&F 12141 | 80 | 100 | 100 |
| Penicillin V | 10 | 100 | 80 |
| Penicillin V | 20 | 100 | 100 |
| Penicillin V | 40 | 100 | 100 |
| Penicillin V | 80 | 100 | 100 |
| Virulence control | – | 20 | 0 |

*Animals treated 1 and 18 hr after infection; 10 mice per group.

effect, however, is obtained with dosages two to four times greater than those of penicillin V (Table 6).

In streptococcal infections, SK&F 12141 protects 70% of the infected animals at dose levels of 5 mg/kg. At this same level, penicillin V protects 100% of the infected animals. DMP, because of lack of oral absorption, fails to produce protection at this level (Table 7).

TABLE 7. *Oral efficacy of SK&F 12141 in mice infected with Streptococcus pyogenes**

| Test compound | Oral dose | Survival after infection | | |
|---|---|---|---|---|
| | | 1 day | 4 days | 7 days |
| | *mg/kg* | % | % | % |
| SK&F 12141 | 5 | 100 | 70 | 70 |
| SK&F 12141 | 10 | 100 | 90 | 80 |
| SK&F 12141 | 20 | 100 | 100 | 100 |
| DMP | 5 | 100 | 0 | 0 |
| DMP | 10 | 100 | 50 | 50 |
| DMP | 20 | 100 | 80 | 80 |
| Penicillin V | 5 | 100 | 100 | 100 |
| Penicillin V | 10 | 100 | 100 | 100 |
| Penicillin V | 20 | 100 | 100 | 100 |
| Virulence control | – | 100 | 20 | 20 |

*Animals treated 1 and 24 hr after infection; 10 mice per group.

SK&F 12141 produces 100% protection against intravenous staphylococcal infections produced by penicillin-resistant staphylococci with dosages of 300 to 500 mg/kg. Penicillin V does not produce this

TABLE 8. *Oral efficacy of SK&F 12141 in mice infected with penicillin-resistant Staphylococcus aureus**

| Test compound | Oral dose | Survival after infection | | |
|---|---|---|---|---|
| | | 4 days | 7 days | 10 days |
| | *mg/kg* | % | % | % |
| SK&F 12141 | 100 | 90 | 50 | 30 |
| SK&F 12141 | 300 | 100 | 100 | 100 |
| SK&F 12141 | 400 | 100 | 80 | 80 |
| SK&F 12141 | 500 | 100 | 100 | 100 |
| Penicillin V | 100 | 50 | 20 | 10 |
| Penicillin V | 300 | 90 | 60 | 40 |
| Penicillin V | 400 | 90 | 70 | 40 |
| Penicillin V | 500 | 90 | 70 | 40 |
| DMP | 100 | – | – | – |
| DMP | 300 | 70 | 40 | 0 |
| DMP | 400 | – | – | – |
| DMP | 500 | 50 | 20 | 0 |
| Virulence control | – | 0 | 0 | 0 |

*Animals treated 3, 24, and 48 hr after infection; 10 mice per group.

effect in any level up to 800 mg/kg. DMP, as expected does not show therapeutic effects since it is not orally absorbable (Table 8).

## Discussion

SK&F 12141 (2-biphenylylpenicillin) has been found to have an antibacterial spectrum generally similar to those of other penicillins. It effectively inhibits penicillin-resistant strains of staphylococci and is resistant to destruction by staphylococcal penicillinase. The anti-staphylococcal activity is not markedly affected by inoculum size.

This penicillin has been found to be orally active. Oral administration of the compound produces a therapeutic effect in mice infected with either *S. pyogenes* or *D. pneumoniae*. This effect is not of the magnitude of penicillin V at the same levels. Oral administration of SK&F 12141 produces a good therapeutic effect in mice infected with penicillin-resistant staphylococci. This effect is superior to penicillin V at all levels tested.

The stability of SK&F 12141 in gastric fluid of pH 1.6 is the same as that of penicillin V. It is 76% bound in pooled human serum and it induces staphylococcal penicillinase.

## Literature Cited

Geronimus, L. H., and S. Cohen. 1957. Induction of staphylococcal penicillinase. J. Bacteriol. **73**:28-34.

Grove, D. C., and W. A. Randall. 1955. Assay methods of antibiotics, a laboratory manual. Medical Encyclopedia, Inc., New York.

Henry, R. J., and R. D. Housewright. 1947. Studies on penicillinase. II. Manometric method of assaying penicillinase and penicillin, kinetics of the penicillin-pencillinase reaction, and the effects of inhibitors on penicillinase. J. Biol. Chem. **167**:559-571.

Kunin, C. M. 1961. Serum binding, distribution and excretion of four penicillin analogues following intravenous injection in man. Proc. Soc. Exptl. Biol. Med. **107**:337-341.

Moat, A. G., L. N. Ceci, and A. Bondi. 1961. Effect of cephalosporin C and various penicillin derivatives on staphylococcal pencillinase and pencillinase-producing staphylococci. Proc. Soc. Exptl. Biol. Med. **107**:675-677.

# A NEW SEMISYNTHETIC PENICILLIN, SK&F 12141

## II. Pharmacological Studies

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SK&F 12141, 2-biphenylylpenicillin sodium salt, demonstrates good antibacterial properties in in vitro laboratory tests and resists destruction by staphylococcal penicillinase. More important, it is effective when administered orally to mice infected with penicillin-resistant *Staphylococcus aureus*.

Pharmacological studies were done to determine oral-absorption rates in mice and dogs and tissue distribution patterns in dogs. Comparative studies with 2,6-dimethoxyphenylpenicillin (DMP) and phenoxymethylpenicillin (penicillin V) were run simultaneously.

Acute and subacute toxicity and intramuscular irritancy studies are also reported here.

### Materials and Methods

*Oral absorption.* Mice weighing 18 to 20 g received oral doses of 200 mg/kg of SK&F 12141, DMP, and penicillin V. At 15 and 30 min after treatment, four mice from each group were killed by bleeding from the heart. The blood samples from each group of four were pooled and diluted in cold phosphate buffer (pH 6). Filter paper discs 13 mm in diameter were moistened in the diluted blood samples and placed on two agar plates seeded with the test organisms, *Sarcina lutea* and penicillin-resistant *S. aureus*. After suitable incubation periods, zones of inhibition were measured.

A triple crossover study was done on three dogs. All received oral doses of 20 mg/kg of each of the three test compounds, SK&F 12141, DMP, and penicillin V. Blood samples, collected at appropriate intervals, were centrifuged, the serum removed, and stored at -20 C until assayed. The standard cup-plate method of Grove and Randall (1955), with *S. lutea* as the test organism, was used to determine the concentration of antibiotics in the serum. The values

were calculated by reference to a standard curve constructed from each of the antibiotics studied.

*Tissue and body fluid distribution following oral and intramuscular administration.* Homogenized suspensions of organs and body fluids of dogs were assayed after oral and intramuscular administration of SK&F 12141, DMP, and penicillin V. Oral dosage consisted of two administrations of 60 mg/kg of the test material, 2 hr apart. Intramuscular dosage consisted of two injections of 30 mg/kg, 2 hr apart. The test animals were killed 1 hr after the second dose, and tissue and body fluids were removed and stored at -20 C until assayed. Each test group consisted of three animals.

*Acute toxicity.* Mice weighing 20 to 26 g were given single oral doses of intravenous injections of SK&F 12141 solubilized in distilled water. The animals were observed for the following 6 days. The $LD_{50}$ values were calculated by the method of Litchfield and Wilcoxon (1949).

*Subacute toxicity.* Three males and three female beagles received intramuscular injections of SK&F 12141. The dogs received daily injections 5 days a week for 4 weeks. One male and one female served as untreated controls.

On the first day of the study, the test animals were injected intramuscularly with 100 mg/kg of an early batch of SK&F 12141 as a 50% solution. They exhibited signs of severe pain. Accordingly, the dose was reduced to 60 mg/kg, as a 30% solution, for the remainder of the study. No evidence of pain was noted at this level.

The animals were observed daily for any gross effects. The following laboratory studies were done at the beginning and end of the test period: (i) hematological studies (total and differential leucocyte count, hemoglobin, hematocrit, and coagulation time); (ii) liver function (bromsulphalein excretion, alkaline phosphatase, and glutamic-pyruvic transaminase); (iii) kidney function (blood urea nitrogen); (iv) carbohydrate metabolism (fasting blood glucose); and (v) electrocardiogram.

At the end of the study, all dogs were killed. Histological examination was done on sections of heart, lungs, liver, intestines, stomach, pancreas, thymus, spleen, kidneys, thyroid, adrenals, testes or ovaries, peripheral nerve, bone and marrow, brain, spinal cord, eyes, and injection sites.

*Intramuscular irritancy.* Three concentrations (25, 40, and 50%) of parenteral grade SK&F 12141 were studied in six dogs. Each concentration, in 1 ml of distilled water, was injected into two dogs. Observations for local and systemic effect were made for 48 hr, when all animals were killed and histological sections of the injection sites were made.

## Results

*Oral absorption.* Pooled blood samples of mice receiving oral doses of SK&F 12141 show the compound to be orally absorbed, since both *S. lutea* and penicillin-resistant *S. aureus* are inhibited with dilutions of the pooled animal blood (Table 1). Penicillin V is also well absorbed by the oral route; however, as would be expected, no activity against the resistant organism is demonstrated. The poor oral absorption of DMP is shown by very small zones of inhibition against *S. lutea* and no zones against resistant staphylococci.

TABLE 1. *Oral absorption in mice**

| Test compound | Test organism | Inhibition zones (mm) | | | |
|---|---|---|---|---|---|
| | | 15-Min blood dilution | | 30-Min blood dilution | |
| | | 1/5 | 1/10 | 1/5 | 1/10 |
| SK&F 12141 | *S. lutea* | 36 | 31 | 34 | 31 |
| DMP | *S. lutea* | 16 | 0 | 0 | 0 |
| Penicillin V | *S. lutea* | 38 | 35 | 38 | 37 |
| SK&F 12141 | Penicillin-resistant *S. aureus* | 20 | 14 | 20 | 18 |
| DMP | Penicillin-resistant *S. aureus* | 0 | 0 | 0 | 0 |
| Penicillin V | Penicillin-resistant *S. aureus* | 0 | 0 | 0 | 0 |

*Oral administration of 100 mg/kg doses. Animals were killed 15 and 30 min after treatment.

Comparison of average blood levels (Table 2), obtained after oral doses of SK&F 12141, DMP, and penicillin V in dogs, indicates that SK&F 12141 is absorbed orally to give serum levels that are slightly lower than penicillin V at the peak level (1 hr) after administration. SK&F 12141 levels can be detected for 3 hr, although those of penicillin V last for 4 hr. DMP does not produce measurable serum levels at any of the sampling times.

TABLE 2. *Average concentration in canine serum after 20 mg/kg oral dose**

| Compound | Average serum levels (μg/ml) | | | | | |
|---|---|---|---|---|---|---|
| | 0.5 hr | 1 hr | 2 hr | 3 hr | 4 hr | 5 hr |
| SK&F 12141 | 0.37 | 1.74 | 0.78 | 0.21 | 0 | 0 |
| DMP | 0 | 0 | 0 | 0 | 0 | 0 |
| Penicillin V | 0.72 | 2.14 | 1.52 | 0.57 | 0.17 | 0 |

**Sarcina lutea* assay.

*Tissue and body fluid distribution following oral and intramuscular administration.* A comparison of the average levels (Table 3), obtained by oral doses of 60 mg/kg of SK&F 12141, DMP, and penicillin V in dogs, shows that SK&F 12141 is absorbed to produce measurable concentrations in the whole blood, serum, kidney, lung,

liver, and bile. Only the liver and bile levels of SK&F 12141 and penicillin V are equivalent. Predictably, DMP shows little or no absorption.

TABLE 3. *Average concentrations in canine body fluids and tissues after oral doses of test compounds (μg/ml)*

| Compound | Whole blood | Serum | Cerebrospinal fluid | Bile | Brain | Kidney | Liver | Lung | Heart |
|---|---|---|---|---|---|---|---|---|---|
| SK&F 12141 | 3.8 | 3.0 | <0.13 | 586 | <0.13 | 10.7 | 1.4 | 0.9 | <1.3 |
| DMP | 0.45 | 0.52 | <0.15 | 73 | <1.5 | 0.7 | <1.5 | <1.5 | <1.5 |
| Penicillin V | 15.2 | 12.4 | 0.18 | 783 | 0.6 | 24.3 | 1.7 | 7.3 | 3.57 |

Comparison of tissue and body fluid levels produced by the three test compounds when administered intramuscularly (Table 4) at 30 mg/kg shows that SK&F 12141 produces liver and lung levels equivalent to penicillin V but lower than DMP. The level of SK&F 12141 in bile is higher than that of either penicillin V or DMP, and the serum, heart, and kidney levels are lower than those of either of the test compounds. The cerebrospinal fluid levels of SK&F 12141 are lower than those of penicillin V but slightly higher than those of DMP. The levels of SK&F 12141 and DMP in brain are not measurable, while penicillin V is present at low, but detectable, levels. The ratio of serum to whole blood levels shows that penicillin V is equally distributed within serum and erythrocytes, although DMP apparently does not penetrate the cells.

TABLE 4. *Average concentrations in canine body fluids and tissues after intramuscular injection of the test compounds (μg/ml)*

| Compound | Whole blood | Serum | Cerebrospinal fluid | Bile | Brain | Kidney | Liver | Lung | Heart |
|---|---|---|---|---|---|---|---|---|---|
| SK&F 12141 | 11.7 | 8.5 | 0.4 | 4,866 | <1.3 | 33.3 | 2.15 | 5.4 | 1.53 |
| DMP | 8.4 | 15.2 | 0.1 | 2,222 | <1.3 | 56.8 | 10.7 | 7.1 | 7.4 |
| Penicillin V | 14.1 | 14.7 | 1.3 | 964 | 0.5 | 56.5 | 2.2 | 5.4 | 2.5 |

*Acute toxicity.* Because the intravenous dose-mortality relationship in mice is not linear, it was not possible to calculate a precise $LD_{50}$ value. The approximate value is between 965 and 980 mg/kg. This value compares favorably with that reported by Glassman et al. (1956) for penicillin G ($LD_{50}$ = 240 mg/kg), but is more toxic than penicillin V ($LD_{50}$ = 2,220 mg/kg). The acute intravenous $LD_{50}$ of DMP is reported by Pindell, Tisch, and Reiffenstein (1961) to be 3,891 mg/kg.

The acute oral $LD_{50}$ for SK&F 12141 is 5,600 mg/kg. The oral $LD_{50}$ for penicillin V is >2,200 mg/kg (Glassman et al., 1956). Oral $LD_{50}$ values for DMP were not available for comparison.

*Subacute toxicity.* No systemic effect in the dogs was observed at any time during the study.

No significant changes in liver function were noted except an increase in pyruvic transaminase in two of six test animals and in one of two control animals.

No changes were observed in kidney function, carbohydrate metabolism, hematological studies, or in electrocardiograms.

The only histological lesion attributable to SK&F 12141 was muscle inflammation at injection sites. All animals showed signs of active muscle regeneration. These muscle lesions appeared less severe than those reported by Hanson (1961) following injections of chlortetracycline and chloramphenicol into rabbit muscle. Furthermore, Hanson made no mention of regeneration of muscle tissue in either instance. Although one cannot be certain because of species difference (dogs vs. rabbits), muscle irritation from SK&F 12141 appears to be less serious than that from the other two antibiotics mentioned.

*Intramuscular irritancy.* No systemic effect, local pain, or tenderness was noted in any of the dogs receiving 250 or 400 mg of SK&F 12141 solubilized in 1 ml of distilled water. One of the two animals receiving 500 mg in 1 ml of distilled water exhibited pain for 2 min after administration of the drug. No pain reaction was observed in the second dog receiving this same concentration (50% solution) of the drug.

Histological examination of the injection sites of 250 and 400 mg/ml solutions revealed no evidence of gross or microscopic changes; however, both dogs receiving the 500 mg/ml solutions showed fairly severe muscle damage on histological examination. Because neither pain nor muscle damage was seen at the two lower (25 and 40%) concentrations of the drug, it is recommended that concentrations of the drug for injection do not exceed 40% (400 mg/ml).

## Discussion

SK&F 12141 (2-biphenylylpenicillin) is orally absorbed to produce tissue and body fluid levels in mice and dogs. These levels are lower than those produced by penicillin V in all fluids and tissues except in liver and bile. All levels are superior to those produced by 2,6-dimethoxyphenylpenicillin.

When SK&F 12141 is injected intramuscularly into dogs, the blood, kidney, and heart levels obtained are slightly lower than penicillin V levels; the lung and liver levels are equivalent to penicillin V levels. The bile level is higher than penicillin V. Comparison with dimethoxyphenylpenicillin shows that SK&F 12141 produces higher levels in whole blood, cerebrospinal fluid, and bile, and lower levels in kidney, liver, lung, and heart.

The acute intravenous $LD_{50}$ in mice is 960 to 980 mg/kg. The oral $LD_{50}$ is 5,600 mg/kg.

Subacute toxicity studies in dogs revealed no systemic effect throughout the test period. The only histological effect attributable to SK&F 12141 injections was muscle inflammation at the site of repeated injection. Additional muscle irritancy tests in dogs injected with various concentrations of more purified drug revealed that muscle inflammation appears with injection of 50% solutions (twice the concentration of the clinical preparation). No inflammation was observed at concentrations of 40% or lower.

## Literature Cited

Glassman, J. M., W. J. Beckfield, E. M. Gore, A. Dervinis, R. Tislow, and J. Seifter. 1956. The toxicologic properties of penicillin V and N,N'-dibenzylenediamine (DBED) dipenicillin V. Antibiotics Ann. 1955-56, p. 534-539.

Grove, D. C., and W. A. Randall. 1955. Assay methods of antibiotics;a laboratory manual. Medical Enclycopedia, Inc., New York.

Hanson, D. S. 1961. Local toxic effects of broad-spectrum antibiotics following injection. Antibiotics & Chemotherapy **6**:390-404.

Litchfield, J. T., Jr., and F. Wilcoxon. 1949. A simplified method of evaluating dose-effect experiments. J. Pharmacol. Exptl. Therap. **96**:99-113.

Pindell, M. H., D. E. Tisch, and J. C. Reiffenstein. 1961. Pharmacological properties of dimethoxyphenylpenicillin, p. 50-57. *In* P. A. Bunn, [ed.], A symposium on the new dimethoxyphenyl penicillin. State University of New York, Syracuse.

# A NEW SEMISYNTHETIC PENICILLIN, SK&F 12141

## III. Laboratory and Clinical Evaluation

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The microbiological and pharmacological studies on SK&F 12141, 2-biphenylylpenicillin, have been described in the two preceding papers. One purpose of this paper is to present data obtained from some absorption-excretion studies done in humans. Another purpose is to report on the clinical efficacy of SK&F 12141 in certain meaningful infections, treated by the senior author, which occur commonly in pediatric practice. These infections are acute otitis media, exudative tonsillitis, and multiple furunculosis. Since most upper respiratory infections are of viral origin and throat cultures are of limited value in determining the etiologic agent, patients with simple pharyngitis were not included in this efficacy study. There is general agreement that most cases of acute otitis media are associated either primarily or secondarily with a bacterial agent. At least, most clinicians will agree that an antibacterial agent is indicated in the treatment of acute otitis media and that effective agents will shorten appreciably the course of this disease and prevent further extension of the infection. The only patients with exudative tonsillitis selected for this clinical evaluation were those from whom a pathogen was isolated, and only those patients with severe furunculosis associated with either a cellulitis or abscess formation were treated with SK&F 12141.

### Materials and Methods

*Oral administration.* A crossover study was done in which five healthy fasting adult subjects ingested single doses of SK&F 12141 ranging from 250 to 2000 mg. The order in which the different doses were taken was randomized. Each dosage was separated by a 3- to 4-day interval. The subjects were bled just prior to drug ingestion and at 0.5, 1, 2, 3, and 6 hr thereafter. The sera were assayed by the standard cup-plate method of Grove and Randall (1955)

using *Sarcina lutea* as the test organism. In addition, serum anti-infective titers were done against both *Streptococcus pyogenes* C203 and a strain of *Staphylococcus aureus* Finland 400, resistant to >1,000 μg/ml of penicillin V. Serial twofold dilutions of the serum samples were made in Trypticase soy broth inoculated with the test organism. After incubation at 37 C for 18 hr, the highest dilution of serum preventing growth of the test organism was recorded as the end point.

Urine was collected for a 12-hr period following drug ingestion. The percentage of the total dose excreted during this time period was calculated from the values obtained in the *Bacillus subtilis* disc-plate assay (Grove and Randall, 1955).

*Intramuscular administration.* Six healthy adult subjects in a crossover study received a single intragluteal injection of SK&F 12141 at two dosage levels, 250 and 500 mg. A 3- to 4-day interval separated the two injections. The subjects were bled just prior to each drug administration and at the following intervals thereafter: 0.5, 1, 2, 3, and 6 hr. Urine was collected for the 12-hr period following the injection. Serum assays, urine assays, and anti-infective titers were carried out according to the methods previously cited in this paper.

*Clinical efficacy.* In testing for clinical efficacy, SK&F 12141 was administered orally either in 500-mg tablets or in a flavored syrup which contained 125 mg/5 ml (1 teaspoon). After some preliminary trial and error therapy, the individual doses were calculated on the basis of 5 to 10 mg/pound of body weight per dose and were given four times daily (Table 1). Since it is known that the absorption of SK&F 12141 is better on a fasting stomach, the parents were instructed to administer the medication 1 hr before each meal and at bedtime. Variation from the recommended dosage regimen occurred in certain instances because of the inability of a child to ingest a tablet or to take the proper amount of the syrup, this deviation always being in the direction of taking less than the prescribed dose. Once the clinical diagnosis of acute otitis media or exudative tonsillitis was made, a throat culture was taken. If the patient had a draining ear or, if myringotomy had to be done, a culture was made

TABLE 1. *Recommended pediatric dosage of SK&F 12141*

| Body weight | Unit dose | | 24 Hr dose |
|---|---|---|---|
| | Milligrams | Form | |
| *lb* | | | *mg* |
| 6 to 12 | 62.5 | 0.5 tsp | 250 |
| 13 to 25 | 125 | 1 tsp | 500 |
| 26 to 60 | 250 | 2 tsp | 1,000 |
| Over 60 | 500 | 1 tab | 2,000 |

from this drainage. Cultures were made directly from the skin lesions in the case of furunculosis. The cultures were sent to the laboratory for bacteriological identification and sensitivity-disc testing with SK&F 12141 and other antibiotics. The penicillin G sensitivity disc contained 1.6 mg and the SK&F 12141 disc contained 5 mg.

Only symptomatic therapy, and no other chemotherapeutic agent, was used concomitantly with SK&F 12141 during the course of the evaluation. The follow-up clinical observation was made 6 to 8 days after therapy was initiated. The clinical results were called "good" or "excellent" if the evidence of infection was minimal or gone at the time of the examination. No attempt was made to obtain repeat cultures or to determine carrier rates.

## Results

*Oral administration.* The average serum concentrations are shown in Table 2 and the anti-infective titers in Table 3. After ingestion of SK&F 12141, 52% of a 1.0-g oral dose was recovered in the urine during the first 12 hr.

TABLE 2. *Average serum concentrations of SK&F 12141 after a single oral dose in five subjects*

| | Average serum concentration (μg/ml) | | | | |
|---|---|---|---|---|---|
| Dosage level | 0.5 hr | 1 hr | 2 hr | 3 hr | 6 hr |
| *mg* | | | | | |
| 250 | 0.66 | 1.66 | 0.44 | 0.067 | 0 |
| 500 | 2.36 | 5.51 | 2.15 | 0.31 | 0 |
| 750 | 5.91 | 6.59 | 1.63 | 0.8 | 0 |
| 1,000 | 6.06 | 16.8 | 3.73 | 1.32 | 0 |
| 2,000 | 34.3 | 28.7 | 8.03 | 4.19 | 0.24 |

TABLE 3. *Average anti-infective titers in human serum after a single oral dose of SK&F 12141 in five subjects*

| | | Time in hr | | | | |
|---|---|---|---|---|---|---|
| Dosage level | Organism | 0.5 | 1 | 2 | 3 | 6 |
| *mg* | | | | | | |
| 250 | *S. aureus* | Undiluted | <1:2* | <1:2 | <1:2 | <1:2 |
| 250 | *S. pyogenes* | 1:18 | 1:33 | Undiluted | <1:2 | <1:2 |
| 500 | *S. aureus* | 1:10 | 1:30 | 1:4 | <1:2 | <1:2 |
| 500 | *S. pyogenes* | >1:64 | 1:67 | 1:70 | 1:3 | <1:2 |
| 750 | *S. aureus* | 1:31 | 1:21 | <1:2 | <1:2 | <1:2 |
| 750 | *S. pyogenes* | 1:516 | 1:420 | 1:62 | 1:20 | <1:2 |
| 1,000 | *S. aureus* | 1:33 | 1:73 | 1:13 | Undiluted | Undiluted |
| 1,000 | *S. pyogenes* | 1:444 | 1:1,260 | 1:127 | 1:36 | <1:2 |
| 2,000 | *S. aureus* | 1:100 | 1:227 | 1:21 | 1:2 | <1:2 |
| 2,000 | *S. pyogenes* | 1:1,452 | 1:1,360 | 1:556 | >1:134 | <1:2 |

*Lowest dilution tested (<).

*Intramuscular administration.* The average serum concentrations and average anti-infective titers are reported in Tables 4 and 5, respectively. During the first 12 hr after injection, 37% of the 250-mg dose and 31% of the 500-mg dose were recovered in the urine.

TABLE 4. *Average serum concentrations of SK&F 12141 after a single intramuscular dose in six subjects*

| | Average serum concentration (μg/ml) | | | | |
|---|---|---|---|---|---|
| Dosage level | 0.5 hr | 1 hr | 2 hr | 3 hr | 6 hr |
| *mg* | | | | | |
| 250 (in 1.0 ml) | 3.12 | 3.23 | 2.52 | 1.34 | 0.20 |
| 500 (in 2.0 ml) | 3.80 | 4.52 | 3.77 | 2.38 | 0.22 |

TABLE 5. *Average anti-infective titers in human serum after a single intramuscular dose of SK&F 12141 in six subjects*

| | | Time in hr | | | | |
|---|---|---|---|---|---|---|
| Dosage level | Organism | 0.5 | 1 | 2 | 3 | 6 |
| *mg* | | | | | | |
| 250 | *S. aureus* | 1:6 | 1:6 | 1:3 | 1:2 | 1:2 |
| | *S. pyogenes* | >1:187 | >1:180 | >1:120 | 1:43 | 1:2 |
| 500 | *S. aureus* | 1:9 | 1:13 | 1:10 | 1:2 | Undiluted |
| | *S. pyogenes* | 1:167 | 1:217 | 1:183 | 1:157 | Undiluted |

*Clinical efficacy.* A total of 52 pediatric patients were treated with SK&F 12141 (Table 6). Of these, there were 37 patients with acute otitis media. The clinical results were "excellent" or "good" in all except one patient, who had a *Pseudomonas* in his ear drainage. These successful clinical results included infections caused by *S. pyogenes, Diplococcus pneumoniae,* and both penicillin G-susceptible and -resistant strains of *S. aureus.* Two patients whose throat cultures yielded *Haemophilus influenzae* had a slower but complete clinical recovery from their infections.

All of the 10 patients with exudative tonsillitis had "excellent" or "good" clinical results. Of the pathogens isolated, two patients had hemolytic *S. pyogenes,* two had *D. pneumoniae,* and six had hemolytic *S. aureus* strains, three of which were resistant to penicillin G.

The remaining five were patients with severe furunculosis associated with cellulitis in three cases and with abscess formation in two cases. Hemolytic *S. aureus* was cultured in each instance, three strains being resistant and two susceptible to penicillin G. All five of these patients had an excellent clinical recovery with SK&F 12141, following treatment failures with one or more other antibiotics in the three patients with the resistant strains.

TABLE 6. *Summary of clinical results*

| Diagnosis | No. of patients | Bacteria isolated | Results | | | |
|---|---|---|---|---|---|---|
| | | | Excellent | Good | Fair | Poor |
| Acute otitis media | 4 | *Streptococcus pyogenes* | 3 | 1 | | |
| | 2 | *Diplococcus pneumoniae* | 2 | | | |
| | 11 | *Staphylococcus aureus* | | | | |
| | | (7)Penicillin G-susceptible | 5 | 2 | | |
| | | (4)Penicillin G-resistant | 4 | | | |
| | 2 | *Haemophilus influenzae* | | 2 | | |
| | 1 | *Pseudomonas* | | | | 1 |
| | 17 | Nonpathogens | 10 | 7 | | |
| Exudative tonsillitis | 2 | *Streptococcus pyogenes* | 2 | | | |
| | 2 | *Diplococcus pneumoniae* | 2 | | | |
| | 6 | *Staphylococcus aureus* | | | | |
| | | (3)Penicillin G-susceptible | 2 | 1 | | |
| | | (3)Penicillin G-resistant | 2 | 1 | | |
| Furunculosis | 5 | *Staphylococcus aureus* | | | | |
| | | (2)Penicillin G-susceptible | 2 | | | |
| | | (3)Penicillin G-resistant | 3 | | | |

*S. pyogenes* and *D. pneumoniae* were uniformly susceptible to penicillin G and SK&F 12141. The hemolytic *S. aureus* strains, which were susceptible to penicillin G, were also susceptible to SK&F 12141. The seven penicillin G-resistant isolates were all susceptible to SK&F 12141.

## Discussion

The results of the absorption-excretion studies in healthy adult subjects indicate that SK&F 12141 is well absorbed orally and produces serum concentrations which are in the clinically useful range if doses of 500 mg or greater are given. The duration of these levels suggests that the drug should be given at least every 4 hr. Intramuscular administration of SK&F 12141 produced serum concentrations which were comparable in height and duration with those from oral administration.

The clinical results demonstrate that SK&F 12141 is efficacious against acute otitis media, exudative tonsillitis, and severe furunculosis in pediatric patients. SK&F 12141 was clearly effective against infections caused by hemolytic *S. pyogenes, D. pneumoniae,* and both penicillin G-susceptible and penicillin G-resistant hemolytic *S. aureus*.

## Literature Cited

Grove, D. C., and W. A. Randall. 1955. Assay methods of antibiotics; a laboratory manual. Medical Encyclopedia, Inc., New York.

# LABORATORY AND CLINICAL EVALUATION OF THE ANTIBACTERIAL ACTIVITY OF PENICILLIN SK&F 12141-Z[1]

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A recent addition to the ever-increasing derivatives of 6-aminopenicillanic acid is the new semisynthetic penicillin with the research designation SK&F 12141-Z (2-biphenylylpenicillin sodium salt). This antibiotic was developed by Smith Kline and French Laboratories primarily for oral use. Early laboratory studies indicated that SK&F 12141-Z is active against both penicillin G-resistant and penicillin G-sensitive strains of staphylococci, as well as against other gram-positive bacteria. Like methicillin and the recently synthesized oral penicillin P-12 (5-methyl-3-phenyl-4-isoxazolyl penicillin), both 6-aminopenicillanic acid derivatives, penicillin SK&F 12141-Z was also shown to resist enzymatic degradation by penicillinase.

The laboratory and clinical experiences with penicillin SK&F 12141-Z over the last few months form the basis of this report.

## Laboratory Studies

*In vitro studies.* The comparative minimal inhibitory concentrations (MIC) of penicillin G, phenethicillin, methicillin, penicillin P-12, and penicillin SK&F 12141-Z required for inhibition of 44 strains of staphylococci by the twofold tube dilution technique are recorded in Table 1. Most of these strains were clinical isolates from patients with active infections.

The results of these studies indicate that penicillin SK&F 12141-Z is active against strains of staphylococci resistant to high concentrations of penicillin G and phenethicillin. The MIC of penicillin SK&F 12141-Z against all strains tested was in the range of 0.20 to 0.78 $\mu$g/ml, as compared to 0.20 to 1.56 $\mu$g/ml for penicillin P-12 and 1.56 to 3.12 $\mu$g/ml for methicillin (except for strain 90, which required 12.5 $\mu$g/ml of methicillin for inhibition). This suggests

[1]Now given generic name ancillin.

that, of the penicillinase-resistant penicillins, penicillin SK&F 12141-Z is more active quantitatively in vitro than methicillin in regard to the cultures tested. Penicillin G-sensitive strains (MIC 0.10 to 0.20 units/ml) were equally sensitive to phenethicillin, but higher concentrations of methicillin, penicillin P-12, and penicillin SK&F 12141-Z were required for inhibition.

TABLE 1. *Comparative in vitro sensitivity (MIC) of various staphylococcal strains to penicillin G, phenethicillin, methicillin, penicillin P-12, and SK&F 12141-Z*

| Stock culture | Phage type | Coagulase | Penicillin G | Phenethicillin | Methicillin | Penicillin P-12 | SK&F 12141-Z |
|---|---|---|---|---|---|---|---|
| | | | *units/ml* | *μg/ml* | *μg/ml* | *μg/ml* | *μg/ml* |
| 10 | 80 | + | >50 | | 1.56 | 1.56 | 0.78 |
| 33 | 54/47/6 | + | 12.5 | >50 | 3.12 | 0.78 | 0.78 |
| 34 | 80/81 | + | 25 | 25 | 3.12 | 0.78 | 0.78 |
| 46 | 80/81 | + | >50 | >50 | 3.12 | 0.78 | 0.78 |
| 60 | | + | 3.12 | 1.56 | 3.12 | 0.39 | 0.39 |
| 62 | 29 | + | >50 | 50 | 1.56 | 0.39 | 0.78 |
| 64 | Not phage typable | − | 0.20 | | 3.12 | 1.56 | 0.39 |
| 66 | 80/81 | + | 12.5 | | 3.12 | 0.78 | 0.78 |
| 69 | 52/42B/81 | + | 6.25 | | 3.12 | 0.78 | 0.78 |
| 72 | 47/75/54 | + | >50 | >50 | 3.12 | 0.78 | 0.78 |
| 93 | 80 | + | 50 | 25 | 12.5 | 0.78 | 0.78 |
| 99 | 80/81 | + | >50 | >50 | 3.12 | 0.78 | 0.78 |
| 101 | 80 | | >50 | >50 | 1.56 | 0.39 | 0.78 |
| 115 | 29 | + | 6.25 | 3.12 | 1.56 | 0.78 | 0.78 |
| 116 | 80/81/52A/52 | + | >50 | >50 | 3.12 | 0.78 | 0.78 |
| 119 | Not phage typable | | 0.10 | 0.10 | 1.56 | 0.20 | 0.39 |
| 127 | Not phage typable | + | 50 | >50 | 1.56 | 0.39 | 0.78 |
| 135 | 80/52/52A/29 | + | 6.25 | 6.25 | 3.12 | 0.78 | 0.78 |
| 136 | 55/38/3C | + | 0.10 | 0.10 | 1.56 | 0.39 | 0.78 |
| 139 | 80/81 | + | >50 | >50 | 3.12 | 0.78 | 0.78 |
| 140 | 80/81 | + | >50 | >50 | 3.12 | 0.78 | 0.78 |
| 153 | | | 12.5 | 6.25 | 1.56 | 0.39 | 0.78 |
| 159 | Not phage typable | + | 25 | 3.12 | 3.12 | 0.39 | 0.78 |
| 160 | 52A/79 | + | 0.10 | 0.10 | 1.56 | 0.20 | 0.39 |
| 161 | 80/81 | + | >50 | >50 | 1.56 | 0.39 | 0.78 |
| 209-P | | | 0.10 | 0.10 | 0.78 | 0.10 | 0.20 |
| 215 | 54 | + | 12.5 | 3.12 | 3.12 | 0.39 | 0.39 |
| 283 | 80/81 | + | 50 | 12.5 | 3.12 | 0.39 | 0.78 |
| 290 | 80/81 | + | 3.12 | 3.12 | 3.12 | 0.78 | 0.78 |
| 296 | 80/81 | + | 12.5 | 12.5 | 3.12 | 0.39 | 0.78 |
| 297 | Not phage typable | + | 3.12 | 1.56 | 3.12 | 0.78 | 1.56 |
| 295 | 80/81 | + | 25 | | 3.12 | 0.39 | 0.78 |
| X | | | 50 | 50 | 3.12 | 0.78 | 0.78 |
| 11 | 80 | + | 25 | 6.25 | 1.56 | 0.39 | 0.78 |
| 22 | | + | >50 | 6.25 | 3.12 | 0.78 | 0.78 |
| 26 | 80/81/52A/52 29/79/54/83/75 | + | 0.10 | 0.10 | 1.56 | 0.39 | 0.39 |
| 42 | 79 | + | 12.5 | 0.78 | 1.56 | 0.39 | 0.39 |
| 56 | 80 | + | 6.25 | 0.78 | 3.12 | 0.39 | 0.78 |
| 142 | 80/81 | + | >50 | >50 | 3.12 | 0.78 | 0.78 |
| 154 | 80/81 | + | >50 | >50 | 1.56 | 0.78 | 0.78 |
| 155 | Not phage typable | + | 25 | 6.25 | 1.56 | 0.39 | 0.78 |
| 74 | 80/81 | + | 50 | 3.12 | 1.56 | 0.39 | 0.78 |
| 282 | 80/81 | + | 50 | 6.25 | 3.12 | 0.39 | 0.78 |
| 138 | 80/81 | + | >50 | >50 | 3.12 | 0.78 | 0.78 |

*In vivo studies.* Cellophane bags containing 0.5 ml of a $10^{-6}$ dilution (1000 organisms/ml) of a 24-hr culture of *Staphylococcus*

*aureus* in heart infusion broth were implanted in the peritoneal cavities of two groups of mice. In one, the Hale strain, which was resistant in vitro to penicillin G (MIC > 50 units/ml) and sensitive to penicillin SK&F 12141-Z (MIC 0.78 μg/ml), was used; in the other, the Read strain, which was sensitive to both penicillin G (MIC 0.10 units/ml) and penicillin SK&F 12141-Z (MIC 0.39 μg/ml), was used. Both groups of mice were treated with penicillin G and penicillin SK&F 12141-Z at the time of implantation of the cellophane bags. The antibiotics were administered orally by intubation in single doses of 0.1, 1.0, 10.0, 50.0, 100.0, and 1000.0 mg/kg, three animals receiving each dose. The mice were killed 48 hr later and the material in the cellophane bags cultured. All of the bags inoculated with the penicillin G-sensitive strain (Read strain) were sterilized by doses of 50 mg/kg of penicillin G and by 100 mg/kg of penicillin SK&F 12141-Z. The penicillin G-resistant strain (Hale strain) failed to grow when doses of 50 mg/kg or greater of penicillin SK&F 12141-Z were utilized, but only the largest dose of penicillin G (1000 mg/kg) inhibited growth (Table 2).

TABLE 2. *Comparative effect of orally administered penicillin G and penicillin SK&F 12141-Z on the sterilization of bacteria-containing cellophane bags in mice**

| | Penicillin G | | SK&F 12141-Z | |
|---|---|---|---|---|
| Oral dose | Hale strain | Read strain | Hale strain | Read strain |
| *mg/kg* | | | | |
| 0 | ++++ | ++++ | ++++ | ++++ |
| 0.1 | ++++ | ++++ | ++++ | ++++ |
| 1.0 | ++++ | ++++ | ++++ | ++++ |
| 10 | ++++ | ± | ++++ | +++- |
| 50 | ++++ | - | - | + |
| 100 | ++++ | - | - | - |
| 1000 | - | - | - | - |

*Symbols used: ++++, normal growth of test organism; ±, slight inhibition; -, inhibition.

The results of this experiment suggest that penicillin SK&F 12141-Z is effective in inhibiting the growth of a penicillin G-resistant strain of *Staphylococcus*, when the antibiotic is administered orally in appropriate doses.

## Clinical Studies

Penicillin SK&F 12141-Z was administered in the treatment of 22 patients with various infections. Pertinent clinical data are presented in Table 3.

TABLE 3. *Summary of clinical data on patients treated with SK&F 12141-Z*

| Diagnosis | Patient, age | Comment on therapy | Total dose and dosage schedule | Response |
|---|---|---|---|---|
| *Staphylococcal infections* | | | | |
| Furunculosis of face | L. I. 26 | Previously treated with tetracycline for 5 days and methicillin for 2 days | 28.0 g 1.0 g q.i.d. p.o. × 7 days | Good response to methicillin. Later continued clearing on SK&F 12141-Z, but subsequently relapsed and had to be retreated with methicillin |
| Recurrent chronic furunculosis | T.K. 53 | Previous therapy included methicillin for 1 week, tetracycline, novobiocin, and probably other antibiotics | 120.0 g 0.5 g.q.i.d. 0.9 × 50 days | Prompt regression of lesions with no recurrences to date. Weekly hemograms and urinalyses normal |
| Recurrent furunculosis | B.S. 26 | Previous therapy with tetracycline for 3 weeks | 120.0 g 1.0 g q.i.d. p.o. | Patient still receiving treatment |
| Recurrent furunculosis | J.S. 30 | No recent previous therapy | 120.0 g 1.0 g. q.i.d. p.o. × 7 days; 0.5 g q.i.d. | Patient still receiving treatment |
| Acute and chronic furunculosis, cellulitis of face | M.S. 49 | Allergic reaction to P-12 after 8 days treatment. No previous reaction to penicillin | 120.0 g 1.0 g q.i.d. p.o. | Patient still receiving treatment |
| Pyoderma of hands and knees | D.R. 12 | No previous therapy | 14.0 g 0.5 g q.i.d. p.o. × 7 days | Clearing of lesions |
| Cellulitis of ear (severe) | R.W. 41 | No previous therapy | 32.0 g 1.0 g q.i.d. p.o. × 2 days; 0.5 g q.i.d. × 12 days | Good response to treatment, clearing of cellulitis |
| Chronic ulcer of buccal mucosa | G.C. 53 | Previous therapy with multiple antibiotics | 28.0 g 0.5 g q.i.d. p.o. × 14 days | Gradual improvement |

| | | | | |
|---|---|---|---|---|
| Infection of aortic graft | G.B.<br>48 | Previous continuous therapy with methicillin for 2 months; patient continued on oral SK&F 12141-Z | 56.0 g<br>1.0 g q.i.d.<br>p.o. × 14 days | Infection controlled with methicillin. Continued healing with SK&F 12141-Z |
| Recurrent furunculosis | J.K.<br>49 | Previous therapy with penicillin and erythromycin. No allergy to penicillin | 6.0 g<br>0.5 g q.i.d.<br>p.o. × 3 days | Partial regression with therapy; patient developed a maculopapular rash 48 hr after institution of therapy. Presently on methicillin, with no allergic reaction |
| Pneumonia | J.T.<br>55 | No previous therapy | 20.0 g<br>1.0 g initial loading dose;<br>0.5 g q.i.d.<br>p.o. × 10 days | Culture of sputum grew *S. aureus*. Prompt clinical response. Course compatible with diagnosis of pneumococcal pneumonia |
| Pneumonia | C.H.<br>29 | No previous therapy | 22.0 g<br>1.0 g q.i.d.<br>p.o. × 1 day<br>0.5 g q.i.d.<br>p.o. × 9 days | Culturé of sputum grew *S. aureus*. Asymptomatic after 2 days. Clearing of pneumonic infiltrate. Response compatible with pneumococcal pneumonia |
| Septicemia | D.B.<br>17 | Previously treated with aqueous penicillin for 4 days | 30.0 g<br>0.5 g q. 4 hr<br>I.M. × 10 days;<br>0.5 g q. 4 hr<br>p.o. | Gradual defervescence over a 4-day period. Clinically improved; pain at site of injection; developed a transient maculopapular rash on 6th day of therapy. *Still receiving treatment* |
| *Gonococcal infections* | | | | |
| Urethritis and arthritis | E.V.<br>29 | No previous therapy | 10.0 g<br>0.5 g q. 6 hr<br>I.M. × 5 days | Prompt clinical improvement. Gram-negative intracellular diplococci on direct urethral smear; culture negative. Patient complained of pain at sites of injection |
| Urethritis and arthritis | P.D.<br>18 | No previous therapy | 50.0 g<br>0.5 g q. 6 hr<br>I.M. × 7 days;<br>0.5 g q. 6 hr<br>p.o. × 3 days | Patient became afebrile on 3rd day of treatment. Smear of urethral and vaginal discharge negative. Patient complained of pain at sites of injection |

(Continued on next page)

TABLE 3. (continued)

| Diagnosis | Patient, age | Comment on therapy | Total dose and dosage schedule | Response |
|---|---|---|---|---|
| *Streptococcal infections* | | | | |
| Pharyngitis and tonsillitis | J.N. 22 | No previous therapy | 15.0 g 1.0 g initial loading dose p.o. 0.5 g q.i.d. p.o. × 7 days | Good clinical response |
| Pharyngitis | M.A. 20 | No previous therapy | 15.0 g 1.0 g initial loading dose p.o. 0.5 g q.i.d. p.o. × 7 days | Prompt clinical response |
| Recurrent sinusitis | R.F. 35 | Previously treated with erythromycin and local irrigation with neomycin | 15.0 g 0.5 g q.i.d. p.o. | *Still on therapy* but improved to date; experienced heartburn with oral ingestion of drug; previous culture of sinuses revealed *Klebsiella* |
| Acute tonsillitis | E.R. 28 | No previous therapy | 15.0 g 0.5 g q.i.d. p.o. × 7 days | Good response to therapy |
| Acute tonsillitis | T.C. 18 | No previous therapy | 15.0 g 0.5 g q.i.d. p.o. × 7 days | Prompt clinical response |
| Tonsillitis | S.S. 20 | No previous therapy | 15.0 g 1.0 g initial dose p.o. 0.5 g q.i.d. p.o. × 7 days | Prompt clinical response |
| Acute tracheobronchitis and pneumonitis | J.L. 69 | No previous therapy | 26.0 g 1.0 g q.i.d. p.o. × 3 days; 0.5 g q.i.d. p.o. × 7 days | Symptomatic improvement within first 3 days of therapy. Gradual clearing of RLL infiltrate with minimal residual pleural thickening |

The number of patients in each major disease category was as follows: furunculosis, 6; tonsillitis and pharyngitis, 5; pneumonia, 3; urethritis, 2; soft-tissue infections, 4; infected aortic graft, 1; sinusitis, 1; and septicemia, 1. The causative organism in 13 of these patients was *Staphylococcus aureus;* in 7, it was *Streptococcus;* and in 2, it was *Gonococcus*.

Of these patients, 17 have completed their course of therapy, and the remaining 5 are still receiving treatment. Penicillin SK&F 12141-Z was administered by the oral route in 20 patients, and by the intramuscular route in 3 patients. The daily dosage was in the range of 2.0 to 4.0 g by the oral route, and 2.0 to 3.0 g by the intramuscular route. The duration of therapy was 7 to 10 days in mild infections, but was usually 1 month in the patients with recurrent furunculosis or other chronic staphylococcal infections. Since the oral route was the main one utilized, the infections selected for treatment were mostly mild to moderately severe cases of recurrent furunculosis, soft-tissue infections, pharyngitis, tonsillitis, and respiratory infections. The total dose was in the range of 10.0 to 120.0 g.

Cases of streptococcal tonsillitis and pharyngitis, soft-tissue infections, gonococcal urethritis, and pneumonia (probably pneumococcal) showed good response to therapy with penicillin SK&F 12141-Z. In the cases of furunculosis, penicillin SK&F 12141-Z caused clearing of lesions in two patients who had no previous therapy. One patient, treated for 7 days, relapsed after discontinuing the SK&F 12141-Z; this patient was then treated with methicillin, which he had received earlier. One patient, who had received methicillin, tetracycline, and novobiocin previously, showed regression of lesions with penicillin SK&F 12141-Z treatment and had no recurrence. Three patients are still undergoing treatment. One patient with an infected aortic graft, treated initially with methicillin (with clinical response), was changed to oral penicillin SK&F 12141-Z to facilitate long-term therapy; this patient has remained clinically well.

The only seriously ill patient treated was a 17-year-old boy with septicemia and metastatic abscesses due to a penicillin G-resistant *Staphylococcus*. He received penicillin G for 4 days without response. He was then given penicillin SK&F 12141-Z by the intramuscular route in doses of 0.5 g every 4 hr; he showed gradual improvement. After 10 days, the intramuscular administration was discontinued, and the same amount was administered orally. The patient is now clinically and bacteriologically well.

No serious side effects were observed in the patients treated with penicillin SK&F 12141, but two patients developed a maculopapular rash. In one patient, this was transient and disappeared without discontinuation of the drug. In the other patient, penicillin SK&F 12141-Z was discontinued; therapy was changed to methicillin. No clinical history of allergy to penicillin G or methicillin was

obtained in either case. One patient gave a history of allergy to penicillin P-12, but had no allergic reaction while receiving penicillin SK&F 12141-Z. No intolerance to the oral administration of the drug was observed among the patients treated, except for one patient who experienced mild heartburn. All three patients who received the drug by the intramuscular route experienced pain at the site of injection, without any local manifestation of inflammation.

Acknowledgment

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# IN VITRO COMPARISON OF VARIOUS PENICILLIN DERIVATIVES WITH PENICILLIN G AGAINST STAPHYLOCOCCI

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Despite the availability of a number of antibiotics which are effective against both penicillin G-resistant as well as penicillin G-sensitive strains of staphylococci, the general usefulness of each of these drugs is limited by the rapid emergence of resistant strains, by difficulty in administration, or by various manifestations of drug toxicity. In addition, many are bacteriostatic rather than bactericidal in activity. Following the isolation of 6-aminopenicillanic acid by Batchelor et al. (1959), semisynthetic penicillins have been produced which vary in a number of characteristics including spectrum of antibacterial activity, acid-stability, and susceptibility to staphylococcal penicillinase. Methicillin was the first of the semisynthetic penicillins which demonstrated marked resistance to staphylococcal penicillinase. However, as first presented by Rolinson et al. (1960) and by many investigators in a subsequent symposium (1961), methicillin was less active than penicillin G against sensitive organisms and had to be administered parenterally. Both of these characteristics limited its general usefulness. Subsequently, additional semisynthetic penicillins, 5-methyl-3-phenyl-4-isoxazolyl penicillin (P-12), 2-biphenylylpenicillin sodium salt (SKF 12141-Z), and 6-(2-ethoxy-1-naphthamido) penicillanic acid (WY-3277), have been produced which would appear to circumvent certain of the shortcomings of methicillin. Specifically, they are more active on a weight basis than methicillin. Yet, in common with methicillin, these semisynthetic penicillins are relatively resistant to staphylococcal penicillinase, hence clinical isolates of staphylococci which are resistant to penicillin G, penicillin V, or phenethicillin should remain sensitive to these agents. Three of these semisynthetic penicillins were compared in vitro with penicillin G and methicillin against clinical isolates of staphylococci.

## Materials and Methods

Strains of *Staphylococcus aureus* were isolated from patients with active infections. Specimens were cultured in Trypticase soy broth (Baltimore Biological Laboratories, BBL) media, then subcultures transferred to "110" media (BBL) for purification. Coagulase tests were performed by the slide method using rabbit plasma.

The following penicillins were utilized: Standard USP sodium penicillin G reference standard; methicillin, lot no. 60L219; P-12 (Bristol), lot no. 61L1188-AFM; SKF 12141-Z (Smith, Kline and French), lot no. 108686; and WY-3277 (Wyeth), lot no. 211-A-E8-8.

Antibiotic sensitivities were performed on the fresh isolates by agar dilution plate technique in which twofold serial dilutions of the penicillins were incorporated into Trypticase soy agar (BBL) with concentrations ranging from 0.312 $\mu$g/ml to 80 $\mu$g/ml. The plates were inoculated with a cotton swab moistened with the suspension of staphylococci to be tested. The staphylococcal strains were grown for 18 hr at 37 C, in Trypticase soy broth. The overnight growth ($10^8$ viable organisms/ml) and a $10^{-3}$ dilution ($10^5$ viable organisms/ml) were used for sensitivity studies. The plates were incubated at 37 C for 24 hr and minimal inhibitory concentrations (MIC) read to the plate showing no visible growth. In vitro sensitivity to other antistaphylococcal agents was performed by agar dilution plate sensitivities with concentrations ranging from 0.312 $\mu$g/ml to 20 $\mu$g/ml. The antibiotics evaluated were: streptomycin, tetracycline, chloramphenicol, novobiocin, erythromycin, bacitracin, kanamycin, and vancomycin.

## Results

The in vitro comparison of various penicillins against 90 strains of staphylococci, freshly isolated from a variety of clinical infections, is presented in Fig. 1. The studies were performed with $10^5$ and $10^8$ organisms as inocula. At the low inoculum size, 76% of the strains were inhibited by 0.625 $\mu$g/ml or less of penicillin G, whereas with an inoculum of $10^8$ organisms only 39% were inhibited by 0.625 $\mu$g/ml or less. With an inoculum of $10^8$ staphylococci, 45% of the strains required greater than 20 $\mu$g/ml of penicillin G for inhibition.

Methicillin, as previously demonstrated, was less active than penicillin G against penicillin G-sensitive strains; however, even at the higher inoculum size ($10^8$ organisms), 96% of strains were inhibited by 2.5 $\mu$g/ml or less. This observation is in accord with the demonstration that methicillin is relatively resistant to enzymatic degradation by staphylococcal penicillinase.

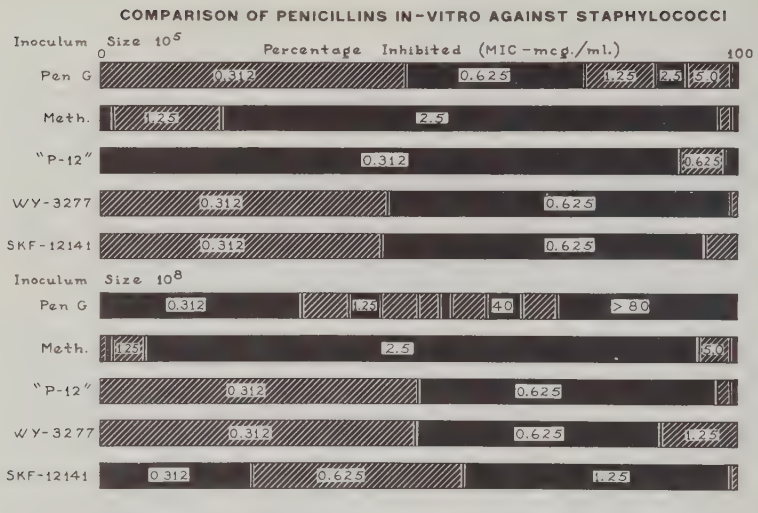


FIG. 1. *Penicillins were tested at concentrations of 0.312, 0.625, 1.25, 2.5, 5.0, 10.0, 20.0, 40.0, and 80.0 μg/ml. Differences in shading represent consecutive twofold dilutions. Percentage of 90 strains inhibited is cumulative from left (0%) to right (100%).*

P-12 was approximately as effective as penicillin G against strains of staphylococci sensitive to penicillin G, with 98% inhibited by $\leq$0.625 μg/ml with the $10^5$ inoculum. In contrast to penicillin G, when the inoculum size was increased to $10^8$ organisms, 96% remained inhibited by $\leq$0.625 μg/ml. P-12 would appear similar to methicillin in its resistance to staphylococcal penicillinase; however, it is more active on a weight basis than methicillin.

With the $10^5$ inoculum, 99% of the strains were inhibited by $\leq$0.625 μg/ml of WY-3277. Again, when the inoculum size was increased to $10^8$ organisms, 88% were inhibited by $\leq$0.625 μg/ml. The remaining strains were inhibited at the 1.25 μg/ml concentration.

With the light inoculum, the in vitro activity of SKF 12141-Z was similar to P-12 and WY-3277, with 95% of strains inhibited by $\leq$0.625 μg/ml. However, with the heavier inoculum ($10^8$ viable units), SKF 12141-Z appeared slightly less active than either P-12 or WY-3277; 57% of strains were inhibited by $\leq$0.625 μg/ml, 42% required a concentration of 1.25 μg/ml for inhibition, and one strain required 2.5 μg/ml. Yet, SKF 12141-Z was far more active than methicillin, which inhibited only 7% of these strains at a concentration of 1.25 μg/ml or less.

A comparison of the susceptibility of 30 of the 91 strains of staphylococci to other potentially effective antistaphylococcal antibiotics is presented in Table 1. The high degree of multiple resistance of these strains is apparent.

TABLE 1. *Antibiotic sensitivities of staphylococci (30 strains)**

| Antibiotic | Resistant† |
|---|---|
| | % |
| Tetracycline | 78 |
| Streptomycin | 67 |
| Bacitracin | 58 |
| Erythromycin | 26 |
| Novobiocin | 20 |
| Chloramphenicol | 9 |
| Kanamycin | 6 |
| Vancomycin | 0 |

*Thirty of the 91 strains were selected for these additional sensitivities.

†Sensitivities by agar dilution plate technique with concentrations of 0.312 to 20 μg/ml. Resistance defined as MIC > 20 μg/ml.

## Discussion

The uniform susceptibility of all strains of staphylococci to P-12, WY-3277, and SKF 12141-Z marks each of these 6-aminopenicillanic acid derivatives as a potent antistaphylococcal drug. Especially interesting were the low concentrations of each required for inhibition in comparison with methicillin. Thus, P-12, WY-3277, and SKF 12141-Z would each appear more active on a weight basis in vitro than methicillin. Based on the observations of Kirby (1945), Geronimus (1950), and Wallmark and Finland (1961), the marked increase in penicillin G resistance shown by comparing small and large inocula was attributed to increased penicillinase activity. Inocula size had very little effect on the susceptibility of staphylococci to methicillin, P-12, or WY-3277 and only a slightly greater effect on susceptibility to SKF 12141-Z, suggesting relatively high resistance to staphylococcal penicillinase.

The antimicrobial activity of a drug constitutes only one facet which needs to be evaluated in determining the potential therapeutic usefulness of the drug. To mention a few factors, differences in acid stability, protein binding, rates of degradation and excretion, as well as local toxic and irritant effects, may vary independently of in vitro antimicrobial activity in broth medium. Hence, these data cannot be interpreted as indicating that P-12, WY-3277, or SKF 12141-Z would be similar in clinical usefulness.

## Acknowledgment

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## Literature Cited

Batchelor, F. R., F. P. Doyle, J. H. C. Nayler, and G. N. Rolinson. 1959. Synthesis of penicillin: 6-aminopenicillanic acid in penicillin fermentations. Nature **183**:257-258.

Geronimus, L. H. 1960. Inoculum size and the apparent sensitivity of staphylococci to penicillins. New Engl. J. Med. **263**:349-351.

Kirby, W. M. M. 1945. Bacteriostatic and lytic actions on sensitive and resistant staphylococci. J. Clin. Invest. **24**:165-169.

Rolinson, G. N., S. Sevens, F. R. Batchelor, J. C. Wood, and E. B. Chain. 1960. Bacteriological studies on a new penicillin - BRL 1241. Lancet **2**:564-567.

Symposium on the new dimethyloxyphenyl penicillin. Early laboratory and clinical experiences with particular reference to resistant staphylococcal disease. 1961. P. A. Bunn, [ed.], State University of New York, Upstate Medical Center, Syracuse.

Wallmark, G., and M. Finland. 1961. Comparative activity of various penicillins against penicillinase producing and non-penicillinase producing staphylococci. Proc. Soc. Exptl. Biol. Med. **106**:78-85.

# DEGREE OF PENICILLINASE RESISTANCE NECESSARY FOR A PENICILLIN TO BE EFFECTIVE AGAINST PENICILLINASE-PRODUCING STAPHYLOCOCCI

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Many new modifications of penicillin have been derived from 6-aminopenicillanic acid, the commercial production of which was made possible by recent findings (Batchelor et al., 1959; Huang et al., 1960). One of these, 2,6-dimethoxyphenyl penicillin, has proven useful in the treatment of benzyl penicillin-resistant staphylococcal infections (Knight et al., 1961). Since most clinically isolated penicillin-resistant strains of *Staphylococcus aureus* owe their resistance to the fact that they contain the enzyme penicillinase (Steinman, 1961*e*), it is presumed that 2,6-dimethoxyphenyl penicillin is effective against such strains by virtue of its very low rate of reaction as a substrate for staphylococcal penicillinase (Steinman, 1961*a*).

In this investigation, a series of penicillins with almost a 1,000-fold range in their reactivities toward staphylococcal penicillinase were tested against large inocula of both penicillinase-producing and nonpenicillinase-producing staphylococci. The effectiveness of a particular penicillin against penicillinase-producing staphylococci appeared to be a function of the enzymatic rate of hydrolysis.

## Materials and Methods

The penicillins examined were kindly supplied by the following sources: Abbott Laboratories (D-4-amino-4-carboxy-*n*-butyl-penicillin); Bristol Laboratories (2,6-dimethoxyphenyl penicillin, 5-methyl-3-phenyl-4-isoxazolyl penicillin, and DL-α-phenoxyethyl penicillin); Eli Lilly and Co. (phenoxymethyl penicillin); Chas. Pfizer & Co., Inc. (6-aminopenicillanic acid, 6-diethylphosphonamido penicillanic acid, and DL-α-phenoxyisobutyl penicillin); Smith, Kline and French Laboratories [7-(D-4-amino-4-carboxy-*n*-butylamino) cephalosporanic

acid]; E. R. Squibb and Sons (benzyl penicillin); and Wyeth Laboratories (2-ethoxy-1-naphthyl penicillin).

The penicillinase used was a cell-free preparation of the intracellular enzyme of *S. aureus* which was released into solution by means of the Nossal bacterial cell disintegrator (Steinman, 1961*d*). the enzyme content having been elevated during growth by induction with 2,6-dimethoxyphenyl penicillin (Steinman, 1961*a*).

The test organisms were clinical isolates of both penicillin-sensitive and penicillin-resistant staphylococci.

*Determination of reactivity towards penicillinase.* The rate of hydrolysis by penicillinase was measured manometrically in the Warburg apparatus through liberation of $CO_2$ from a $NaHCO_3$ buffer system by formation of penicilloic acid (Steinman, 1961*c*). For the rate of hydrolysis of 6-aminopenicillanic acid, however, this procedure is inaccurate and an iodometric assay was employed (Steinman, 1961*b*).

*Determination of minimal inhibitory concentration (MIC).* Minimal growth-inhibitory concentrations of the various penicillins were determined by turbidimetric observations, over a 24-hr period, of the growth rate of cultures in Trypticase soy broth. In this study, where large inocula were used, the starting cultures were derived from overnight cultures which were diluted 1:33 in fresh broth, reincubated for 2 hr at 37 C, and then inoculated at a dilution of 1:10 to yield initial cell concentrations of $10^8$ organisms per ml.

## Results

A number of penicillins were examined for their relative reactivities with staphylococcal penicillinase. Table 1 lists the penicillins arranged according to decreasing rates of hydrolysis, which are given relative to the rate for benzyl penicillin, in the second column. The over-all range in the enzymatic rates of hydrolysis for this series of penicillins was almost 1,000-fold.

The several penicillins were studied also for their antibiotic activities against both nonpenicillinase-producing and penicillinase-producing strains of *S. aureus* (Table 1). It has been regularly observed that the MIC value of a penicillin against a staphylococcal strain which does not produce penicillinase is only slightly affected by a wide variation in the initial concentration of organisms, whereas the MIC value against an enzyme-producing strain is greatly affected by the initial concentration of cells. In this study, the initial cell concentrations were as high as practicable ($10^8$ organisms per ml) to render the effect of inoculum size as large as possible.

As seen from the data in Table 1, except for 6-aminopenicillanic acid and cephalosporin C, which are penicillins of very low activity, the variation in MIC values against the nonpenicillinase-producing strain was of the order of 100-fold, and was unrelated to

reactivity toward penicillinase. In contrast, the MIC values against the penicillinase-producing strain, which covered almost a 100,000-fold range as the result of the very high initial cell concentrations, roughly paralleled the relative enzymatic reactivities.

TABLE 1. *Ratios of minimal inhibitory concentrations against high initial cell concentrations ($10^8$ organisms per ml) of penicillinase-producing versus nonpenicillinase-producing Staphylococcus aureus as compared to reactivities with staphylococcal penicillinase*

| Penicillin | Relative rate of hydrolysis (benzyl penicillin = 100) | MIC ($\mu$g/ml) | | Ratio of MIC values for penicillinase- to nonpenicillinase-producing *S. aureus* |
|---|---|---|---|---|
| | | Nonpenicillinase producing | Penicillinase producing | |
| Phenoxymethyl | 125 | 0.03 | 40,000 | >10,000 |
| Benzyl | 100 | 0.02 | 40,000 | >10,000 |
| DL-α-Phenoxyethyl | 75 | 0.03 | 6,000 | >10,000 |
| D-4-Amino-4-carboxy-*n*-butyl | 50 | 8.0 | 8,000 | 1,000 |
| DL-α-Phenoxyisobutyl | 35 | 0.4 | 400 | 1,000 |
| 6-Diethylphosphonamido penicillanic acid | 15 | 4.0 | 400 | 100 |
| 6-Aminopenicillanic acid | 7.5 | 44.0 | 1,100 | 25.0 |
| 5-Methyl-3-phenyl-4-isoxazolyl | 0.8 | 0.3 | 1.0 | 3.3 |
| 7-(D-4-Amino-4-carboxy-*n*-butylamino) cephalosporanic acid | 0.6 | 88.0 | 220 | 2.5 |
| 2,6-Dimethoxyphenyl | 0.4 | 1.0 | 2 | 2 |
| 2-Ethoxy-1-naphthyl | 0.2 | 0.4 | 0.8 | 2 |

The discrepancy in the parallel relationship between the two activities is due principally to the large variation in the intrinsic antibiotic activities of the penicillins. By taking the ratio of the MIC value against a penicillinase-producing strain to the MIC value against an equal concentration of a nonpenicillinase-producing strain, a number is obtained which is no longer a function of the absolute antibiotic activity of the penicillin. This ratio expresses the relative amount of antibiotic required to inactivate a given concentration of a penicillinase-producing strain versus a nonpenicillinase-producing strain. For penicillins which are attacked by penicillinase, the ratios increased with increased initial cell concentrations. For the large inocula used in these studies, the variation in the ratios was more than 10,000-fold. The ratios directly paralleled the relative rates of enzymatic hydrolysis of the penicillins investigated.

## Discussion

It is evident that the relative effectiveness of a penicillin against penicillinase-producing staphylococci is strongly influenced by its

relative susceptibility to destruction by the enzyme. The minimal growth-inhibitory concentrations of penicillins which were sensitive to penicillinase varied with the concentrations of penicillinase-producing organisms. Furthermore, the ratios of the minimal inhibitory concentrations of such penicillins against high cell concentrations of penicillinase-producing compared to nonpenicillinase-producing staphylococci paralleled the enzymatic rates of hydrolysis. In marked contrast, the several penicillins which were hydrolyzed by staphylococcal penicillinase at rates 100 to 500 times more slowly than benzyl penicillin were all equally effective against both large and small inocula of penicillinase-producing staphylococci. It was observed also that the penicillins refractory to the action of penicillinase were just as effective against penicillinase-producing staphylococci as against nonpenicillinase-producing strains. This indicated that, other than the penicillinase-forming characteristic, there was no inherent difference between the two varieties with respect to their response to the antibiotic action of penicillin.

Since the ratios of the minimal growth-inhibitory concentrations for the two types of staphylococci reached a lower limit of about 2 for the penicillins possessing rates of enzymatic hydrolysis less than 1% that of benzyl penicillin, it is suggested that this value represents the critical degree of resistance necessary for a penicillin to be effective against large numbers of a penicillinase-producing staphylococcus.

## Acknowledgment

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## Literature Cited

Batchelor, F. R., F. P. Doyle, J. H. C. Nayler, and G. N. Rolinson. 1959. Synthesis of penicillin: 6-aminopenicillanic acid in penicillin fermentations. Nature **183**:257-258.

Huang, H. T., A. R. English, T. A. Seto, G. M. Shull, and B. A. Sobin. 1960. Enzymatic hydrolysis of the side chain of penicillins. J. Am. Chem. Soc. **82**:3790-3791.

Knight, V., P. A. Bunn, H. G. Steinman, and P. Fireman. 1961. A new penicillin derivative resistant to penicillinase, dimethoxyphenylpenicillin. Combined Clinical Staff Conference of the National Institutes of Health (March 23, 1961). Antibiotics & Chemotherapy **11**:537-554.

Steinman, H. G. 1961*a*. A biochemical comparison of 6-aminopenicillanic acid, benzylpenicillin, and 2,6-dimethoxyphenylpenicillin. Proc. Soc. Exptl. Biol. Med. **106**:227-231.

Steinman, H. G. 1961*b*. Determination of the rate of enzymatic hydrolysis of 6-aminopenicillanic acid. Federation Proc. **20**:232.

Steinman, H. G. 1961*c*. Factors modifying induced formation of penicillinase in *Staphylococcus aureus*. J. Bacteriol. **81**:895-902.

Steinman, H. G. 1961*d*. Preparation of cell-free extracts of staphylococcal penicillinase. J. Bacteriol. **82**:620-621.

Steinman, H. G. 1961*e*. The role of penicillinase in the penicillin-resistance of *Staphylococcus aureus*. Abstr. 5th Intern. Congr. Biochem., p. 299.

# CHANGES IN NASAL FLORA IN PATIENTS AND CONTACTS WHEN METHICILLIN WAS USED EXTENSIVELY ON HOSPITAL WARDS

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The introduction of a new antibiotic, effective against staphylococci, into general use in a hospital has usually resulted in the appearance of strains of staphylococci resistant to that antibiotic. In general, resistant coagulase-negative staphylococci are detected first (Lepper, *unpublished data*). Later, resistant coagulase-positive strains are found. These resistant strains implant in the anterior nares of hospital personnel and patients in wards where a given antibiotic is in intensive use (Lepper et al., 1954). Discharged patients and off-duty personnel disseminate the strains into the community (Williams et al., 1960).

The mechanism of penicillin G resistance of staphylococci, isolated from infections, has been through elaboration of penicillinase. With the introduction of methicillin, a penicillinase-resistant penicillin has come into general clinical use. Published reports (Smith and Counts, 1960; Rutenberg, Greenberg, and Schweinberg, 1960; Jevons, 1961; Casson and Lannon, 1960; Fedorko and Katz, 1960) indicate that naturally occurring methicillin-resistant strains of staphylococci are rare but do exist. Observations by others (Stewart, 1961) and in our laboratory indicate that staphylococci can be "trained" in the laboratory to grow in successively higher concentrations of methicillin. This resistance is not due to a methicillin-destroying penicillinase. This methicillin resistance can be a genetically stable trait (Dowling, 1961). It would be logical, therefore, to assume that nasal culture surveillance of a patient-personnel population in hospital wards where methicillin was in extensive use would lead to the detection of any resistant strains which might arise.

The present report is part of a study which was designed to detect the emergence of methicillin-resistant staphylococci, to study the effect of methicillin on the nasal carrier state in treated patients, and to gain some knowledge about the clinical effectiveness of the drug.

## Materials and Methods

*Use of methicillin.* From 15 November 1960 through 30 June 1961, all coccal infections on the two medical wards of Illinois Research and Educational Hospitals, with the exception of some cases of endocarditis, were treated with methicillin in appropriate doses.

*Surveillance techniques.* Anterior nasal cultures were obtained on personnel 1 week prior to the inception of the study and at monthly intervals thereafter. Patients were cultured on admission, when possible, and at weekly intervals thereafter. These surveys were 90 to 95% complete.

*Bacteriology.* Routine admission and surveillance cultures were done by swabbing the anterior nares with a sterile cotton swab, which was placed in sterile saline until its transport to the laboratory. The cultures were planted as soon as possible. Admission cultures, at night or on weekends, were taken with alginate swabs and placed in Stuart's medium until planted. Each swab was streaked on a sheep blood agar plate, incubated 24 hr, and representative colonies were subcultured in nutrient broth for coagulase testing, bacteriophage typing, sensitivity testing to methicillin, benzylpenicillin, and tetracycline, and stored in the deep freeze at -20 C. The minimal inhibitory concentration was determined by serial twofold tube dilutions in tryptose phosphate broth. Sensitivity testing was done on all coagulase-positive isolates, all coagulase-negative isolates from treated patients, all coagulase-negative isolates from patients hospitalized for more than 2 weeks, and a random 10% sample of remainder of the coagulase-negative strains.

*Period of study.* The study covered 228 days of observation. During this period one or more patients received methicillin 88% of the time on one ward, 85% on the other, or 96% of the time on one ward or the other. During the winter months of high infection prevalence, periods of continuous usage of 60 days or more were recorded. A total of 80 patients received methicillin during the period of study, or approximately 9% of the total patient population.

*Analysis of data.* Staphylococci were tabulated as resistant if the minimal inhibitory concentration was 7.8 $\mu$g/ml or greater.

In the analysis of carrier status, no individual cultures were tabulated. Tabulations were made by shift or failure to shift from week to week. Changes in nasal culture status were analyzed by week of observation in the study. Weekly changes for the different groups were also combined by group for intergroup comparison. In methicillin-treated patients, determination of the change in the nasal carrier status after the first week of treatment was based on the last pretreatment culture. Patients were considered to have acquired a new coagulase-positive organism if the culture changed from no staphylococci to coagulase-positive, from coagulase-negative to coagulase-positive, or from one coagulase-positive strain to a different

strain. Coagulase-positive strains were judged to be different if they differed by two or more phages within any one phage group or by one phage in another phage group. A change from nontypable to typable or the reverse was also tabulated as a change. Obviously, only patients with two or more cultures could be analyzed for change in nasal flora.

## Results

*Methicillin-resistant isolates.* Sensitivity tests were done on 1,616 staphylococcal isolates, 941 of which were coagulase-positive and 675 coagulase-negative. Of these coagulase-positive strains, 718 were from nasal cultures in patients. The remainder were from personnel. A monthly analysis of the fluctuations in the nasal carrier state of staphylococci in personnel will be reported later. No

TABLE 1. *Resistant staphylococci isolated*

| Classification of carrier | Date of isolation | Type of isolate* | Sensitivity on fresh isolation (M.I.C.) |
|---|---|---|---|
| Nurse no. 003 | 1-17-61 | – | 31.3 (Initial culture) |
| | 2-23-61 | – | 3.9 |
| | 3-16-61 | – | 3.9 |
| | 5-17-61 | – | 3.9 |
| Nurse no. 33 | 11-18-60 | – | 1.95 |
| | 12-15-60 | – | (Did not grow on subculture) |
| | 1-16-61 | – | 31.3 (Third culture) |
| | 2-14-61 | – | 125.0 (Fourth culture) |
| | 3-13-61 | – | 3.9 |
| | 4-19-61 | 0 | |
| | 5-17-61 | 0 | |
| Patient no. 414 | 1-18-61 | – | 1.95 |
| | 1-30-61 | – | 125.0 |
| | 2- 6-61 | – | 62.5 |
| Patient no. 630 | 3-14-61 | – | 3.9 |
| (Received | 3-21-61 | – | 1.95 |
| methicillin | 3-27-61 | – | 62.5 |
| 3/12-3/22) | 4- 3-61 | – | (Did not grow on subculture) |
| Nurse no. 9 | 3-16-61 | – | 0.9 |
| | 4-17-61 | – | 125.0 |
| | 5-15-61 | + | 0.98 |
| | 6-16-61 | – | 1.95 |
| Patient no. 492 | 4-18-61 | 0 | |
| (Received | 4-24-61 | 0 | |
| methicillin | 5- 1-61 | – | 250.0 |
| 3/24-4/24) | 5- 8-61 | – | 7.8 |
| | 5-15-61 | – | 3.9 |

*Symbols used: coagulase-negative (–); coagulase positive (+); no staphylococci (0).

resistant coagulase-positive organisms were isolated by nasal culture during the period of the study, but nine coagulase-negative resistant strains were isolated from three nurses and three patients. All of these isolations were from the same ward.

Table 1 shows the isolates with their sensitivities. Two (no. 630 and 492) of the three patients had received methicillin, but the drug had been discontinued prior to the isolation. Of the group of six individuals, two (nurses no. 33 and 9) had susceptible isolates, prior to and following the resistant isolate. The initial isolates of two others were resistant. One of these (patient no. 492) had two previous cultures negative for staphylococci. The other (nurse no. 003) had a resistant isolate on the initial culture. Both had subsequent susceptible isolates. The remaining two (patients no. 414 and 630) had initial susceptible isolates with resistant strains in the final cultures. The resistant isolates were scattered, from January through May, among patients and personnel on one of the two wards under observation.

We have isolated only one methicillin-resistant staphylococcus associated with clinical infection. This was a coagulase-positive *Staphylococcus aureus,* which was isolated from the blood of a child with congenital heart disease and endocarditis. This patient had been treated with small doses of methicillin for 12 weeks prior to admission.

*Over-all changes in the nasal carrier state of patients.* A total of 2,555 cultures was taken from 882 patients, or an average of 2.9 cultures per patient. Of these, 28.1% contained coagulase-positive staphylococci; 53.8%, coagulase-negative staphylococci; and 18.0%, no staphylococci. Figure 1 presents the percentage of patient-weeks of observation in which nasal acquisition or loss of coagulase-positive staphylococci occurred in each of the following classes of patients: (i) untreated controls, (ii) methicillin-treated patients during the treatment course, (iii) methicillin-treated patients after treatment, (iv) a small group of unselected patients who received tetracyclines, chloramphenicol, or a combination of erythromycin and novobiocin.

In the control group, 233 of 1,375 observations (16.9%) revealed gain of coagulase-positive strains. There were 432 observations of coagulase-positive staphylococci in control patients who had one or more subsequent cultures. This constituted the group exposed to the risk of the loss of coagulase-positive strains. In 131 instances (30.3%) such a loss occurred.

During methicillin treatment, 2 of 92 observations revealed acquisition of coagulase-positive strains. There were 13 opportunities for loss of coagulase-positive staphylococci during methicillin treatment. In eleven instances this occurred, a loss of 85%.

A small number of observations in patients in group 4 revealed acquisition in 6 of 15 (40%) and loss in 3 of 6 (50%).

Following cessation of methicillin treatment, 15 of 100 patients acquired coagulase-positive strains. There were 14 opportunities for loss of coagulase-positive strains. In two instances (14%) such loss occurred.

Statistical analysis, by chi square method of these two groups of data, reveals the abnormal distribution to be highly significant, at the 0.1% level. The chief differences lie in the methicillin-treated group. The postmethicillin treatment and untreated groups do not differ significantly from the expected values or from each other.

Figure 2 shows the loss of coagulase-positive staphylococci, by weeks of observation during methicillin treatment, following methicillin treatment and in untreated controls. Loss of coagulase-positive staphylococci occurred in eight of nine possible instances, or 89%, during the first week of treatment. During the second week three of three coagulase-positive strains were lost (100%). No shifts to coagulase-positive were made on methicillin treatment after the third week, although other changes for acquisition were observed.

Untreated controls revealed loss of 7 of 13 positives in the first week of observation (54%), 53 of 139 (38%) in the second week, 19 of 89 (21%) in the third week, 21 of 78 (26%) in the fourth week, 16 of 47 (34%) in the fifth week, 6 of 21 (29%) in the sixth week, 2 of 12 (17%) in the seventh week, and 1 of 9 (11%) in the eighth week.

Coagulase-positive staphylococci were lost during the first week after methicillin treatment in one of three possible instances. After

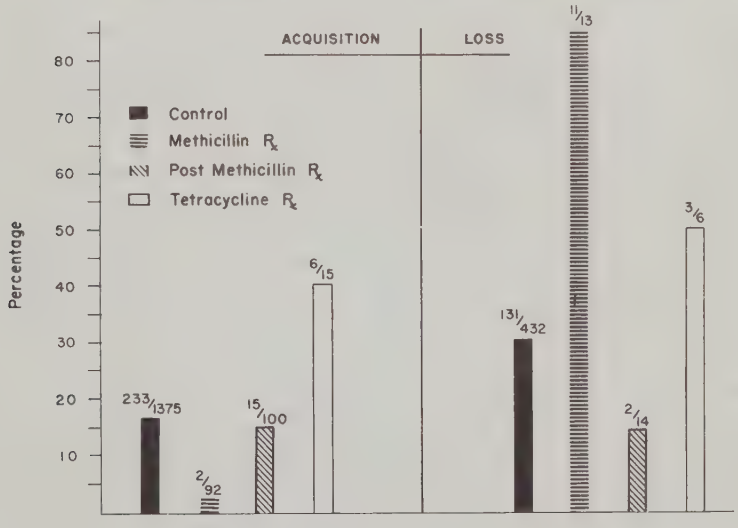


FIG. 1. *Acquisition or loss of coagulase-positive staphylococci per patient-week of observation.*

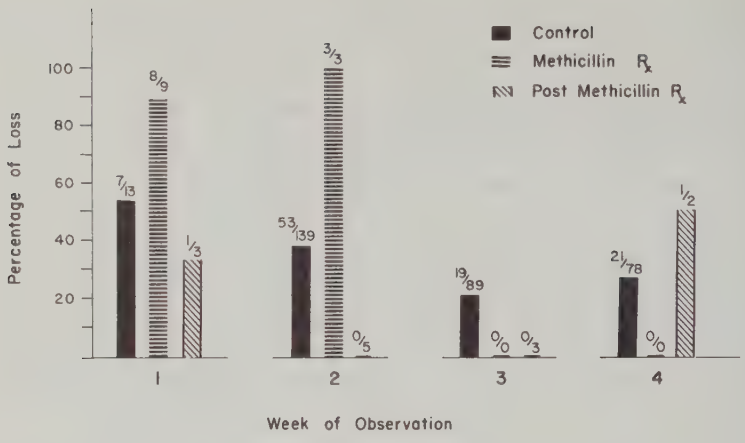


FIG. 2. *Loss of coagulase-positive staphylococci by week of observation.*

the previous statement that no coagulase-positive strains were found after the second week of treatment, it may seem paradoxical that there was the possibility of losing strains in the first post-treatment week. This is accounted for by the fact that several patients received one week or less of treatment; this left three coagulase-positive strains at risk of loss in the first post-treatment week. Subsequent to the first week after treatment, insignificant loss of coagulase-positive strains occurred; 0 to 5 in the second week; 0 of 3 in the third week; and 1 of 2 in the fourth week.

Figure 3 reveals the rate of acquisition by week of observation of coagulase-positive staphylococci in the methicillin-treated, control, and the postmethicillin-treatment groups. In the control group, 18 of 56 observations (32%) revealed gain in the first week; 84 of 497 (17%), in the second week; 59 of 303 (19%), in the third week; 19 of 190 (10%), in the fourth week; 16 of 125 (13%), in the fifth week; 8 of 68 (12%), in the sixth week; 11 of 47 (23%), in the seventh week; and 5 of 28 (18%) in the eighth week. In the methicillin-treated group, in 2 of 36 observations (5.5%), coagulase-positive staphylococci were acquired during the first week of treatment; in the second week, 0 of 37; in the third week, 0 of 10; in the fourth week, 0 of 6; and in the fifth week, 0 of 3. The postmethicillin-treatment group revealed gain of 9 of 47 (19%), in the first week; 2 of 26 (8%), in the second week; 1 of 15 (7%), in the third week, 1 of 7 (14%), in the fourth week; and 1 of 3 (33%), in the fifth week.

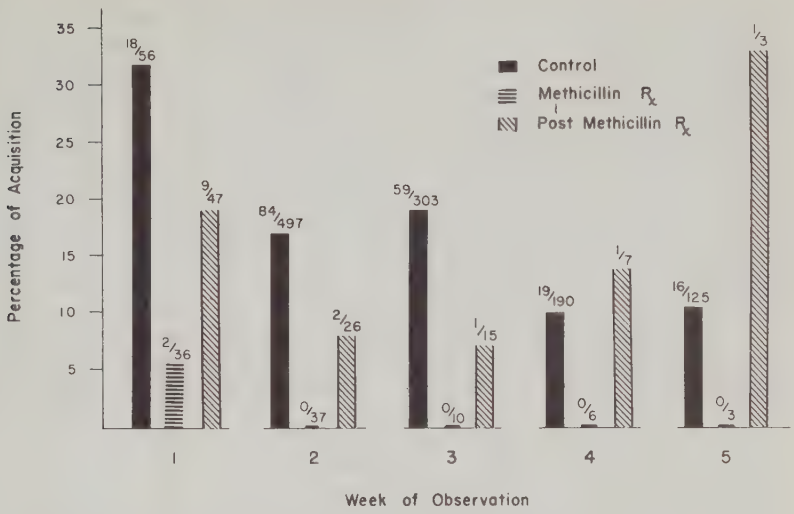


FIG. 3. *Acquisition of coagulase-positive staphylococci by week of observation.*

## Discussion

Intensive search for methicillin-resistant staphylococcal strains has failed to reveal a single coagulase-positive resistant strain in 228 days of observation. Previous observations by the same techniques following the introduction of antistaphylococcal drugs of similar effectiveness would lead one to expect recognition of the emergence of resistant strains in this period of time (Lepper et al., 1957). One might argue that our use of methicillin (in about 10% of all patients) was not intensive enough to evoke the emergence of resistant strains. However, we employed the drug in patients who would have received benzylpenicillin if the study had not been in progress. No general medical ward is likely to use the drug more intensively.

Drugs which suppress the nasal carriage of *S. aureus* should favor the implantation of naturally occurring resistant strains. Acquisition of coagulase-positive staphylococci was revealed in 16.9% of all observations in untreated control patients, while 30.3% of such patients lost their coagulase-positive strain. On methicillin treatment, only 2% of all observations revealed acquisition of a coagulase-positive strain while 85% of coagulase-positive strains were lost on treatment. These differences are significant at the 0.1% level.

During the first few weeks after methicillin treatment, patients resembled the control group in that 15% acquired new strains; however, only a small number (14%) lost strains. The differences between the acquisition-loss rates of the control group and the post-methicillin-treatment group are not statistically significant. Patients under therapy with tetracyclines or other broad-spectrum antibiotics acquired new coagulase-positive staphylococci in 40% of the weeks of observation and lost strains in 50% of instances where they had been present. Although the number of observations was small among patients treated with broad-spectrum antibiotics, they indicate that coagulase-positive staphylococci were being readily exchanged on the wards.

The data indicate a marked supression of the nasal carriage of coagulase-positive staphylococci during methicillin treatment, and rates of acquisition and loss after cessation of therapy similar to rates in untreated controls. However, in spite of this effect on the nasal carrier state, no resistant coagulase-positive strains were isolated. Jevons (1961), working in England, found three of 5,440 strains tested to be nonsusceptible to methicillin. Among 541 strains of *S. aureus* tested by investigators in the United States, seven had minimal inhibitory concentrations in excess of 6 $\mu$g/ml (Casson and Lannon, 1960; Fedorko and Katz, 1960; Rutenberg et al., 1960; Smith and Counts, 1960). These data would indicate that naturally occurring methicillin-resistant strains are rare.

Extrapolations from experience with similar studies on erythromycin, novobiocin, and other antibiotics of comparable effectiveness suggest that resistant strains, if present, should have been selected so that they persisted. This leads us to believe that methicillin-resistant staphylococci are unlikely to be a serious clinical problem. However, more prolonged and intensive use of the drug might lead to the establishment of resistant strains.

Elek and Fleming (1960) sprayed 1.0 g of methicillin into the air of a nursery four times per day. This depressed the nasal colonization rate of coagulase-positive staphylococci in newborn infants from 50%, in the control period, to 2% after six months. They observed no coagulase-positive resistant strains. Most of the infants became colonized with coagulase-negative strains, many of which were not susceptible to methicillin.

The report of Elek and Fleming deals with a situation of more uniform and intense drug exposure than does the present report. We isolated resistant coagulase-negative micrococci from only 2 of 80 treated patients, one other patient contact, and three nurses, making a total of nine resistant coagulase-negative strains among 675 tested. Only four of the individuals had subsequent sensitive coagulase-negative isolates. Only one had a resistant isolate on the first culture—a nurse with a resistant isolate in January 1961 who had worked on the ward for two months previously, but had been missed during the

surveys. The untreated patient with a resistant isolate had been hospitalized for more than a week prior to the recovery of this isolate. No resistant coagulase-negative strains were obtained from admission cultures. Acquisition seemed to occur on the ward, and the carrier state was transient. Carriage of methicillin-sensitive, coagulase-negative staphylococci was quite common in our patients during methicillin treatment. Their persistence raises the question of the interconvertibility of coagulase-positive and -negative strains and the role of the coagulase-negative organism in the perpetuation of the coagulase-positive organism. Among twelve patients who had coagulase-positive strains prior to treatment, three exhibited the same phage type post-treatment. Of these, four acquired different phage types post-treatment and five had coagulase-negative or no staphylococci following treatment. Further elucidation of this problem must await further delineation of "markers" of coagulase-negative strains.

## Acknowledgments

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## Literature Cited

Cassan, K., and J. H. Lannon. 1960. Sensitivity testing of dimethoxyphenyl penicillin. p. 43-49. *In* P. A. Bunn, [ed.], A symposium on the new dimethoxyphenyl penicillin. Early laboratory and clinical experiences with particular reference to resistant staphylococcal disease. State University of New York, Upstate Medical Center, Syracuse.

Dowling, H. F. 1961. The newer penicillins. J. Clin. Pharmacol. Therap. 2:572-579.

Elek, S. D., and P. C. Fleming. 1960. A new technique for the control of hospital cross infection. Experience with BRL 1241 in a maternity unit. Lancet 2:569-572.

Fedorko, J., and S. Katz. 1960. Laboratory comparison of a new synthetic penicillin, dimethoxyphenyl penicillin with alpha phenoxyethyl penicillin and penicillin G, p. 201-205. *In* P. A. Bunn, [ed.], A symposium on the new dimethoxyphenyl penicillin. Early laboratory and clinical experiences with particular reference to resistant staphylococcal disease. State University of New York, Upstate Medical Center, Syracuse.

Jevons, M. P. 1961. Celbenin resistant staphylococci. Correspondence, Brit. Med. J. 1:124-125.

Lepper, M. H., B. Moulton, H. F. Dowling, G. G. Jackson, and S. Kofman. 1954. Epidemiology of erythromycin resistant staphylococci in a hospital population. Effect on therapeutic activity of erythromycin. Antibiotics Ann. 1953-1954, p. 308-313.

Lepper, M. H., H. F. Dowling, G. G. Jackson, H. W. Spies, and M. Mellody. 1957. The effect of the routine use of novobiocin and spiramycin in combination. Antibiotics Ann. 1956-57, p. 640-647.

Rutenberg, A., H. L. Greenberg, and F. B. Schweinberg. 1960. Clinical experiences with dimethoxyphenyl penicillin, p. 101-108. *In* P. A. Bunn [ed.], A symposium on the new dimethoxy-

phenyl penicillin. Early laboratory and clinical experiences with particular reference to resistant staphylococcal disease. State University of New York, Upstate Medical Center, Syracuse.

Smith, I. M., and G. W. Counts. 1960. Laboratory and clinical studies of dimethoxyphenyl penicillin, p. 83-92. *In* P. A. Bunn, [ed.], A symposium on the new dimethoxyphenyl penicillin. Early laboratory and clinical experiences with particular reference to resistant staphylococcal disease. State University of New York, Upstate Medical Center, Syracuse.

Stewart, G. T. 1961. Changes in sensitivity to methicillin. Brit. Med. J. 2:863-866.

Williams, R. E. O., R. Blowers, L. P. Garrod, R. A. Shooter. 1960. Hospital infections, cause and prevention. The Year Book Publishers, Inc., Chicago.

# CURRENT STATUS OF THERAPY WITH THE SEMISYNTHETIC PENICILLINS

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The purpose of this paper is to inform the average practitioner of medicine of the clinical results of 2 years of investigation with the semisynthetic penicillins which, for the most part, are commercially available at the present time.

Phenethicillin, the first of the new penicillins, was found to be well absorbed, give satisfactory blood levels, and to have an acceptable antibacterial activity against many gram-positive organisms. It is not within the scope of this paper to discuss its advantages or disadvantages when compared to penicillin G, but to point out its therapeutic efficacy in the management of penicillin-sensitive gram-positive infections.

I have treated 200 moderate and severe infections with phenethicillin (Table 1). The results are described below.

TABLE 1. *Infections treated with phenethicillin*

| Infection | No. of cases |
|---|---|
| Pneumonia | 55 |
| Tonsillitis | 51 |
| Bronchitis | 32 |
| Sinusitis | 8 |
| Tracheitis | 1 |
| Empyema | 1 |
| Subacute bacterial endocarditis | 18 |
| Skin and soft-tissue infections | 13 |
| Pyelonephritis | 10 |
| Prostatitis | 5 |
| Osteomyelitis | 2 |
| Thrombophlebitis | 2 |
| Pyogenic arthritis | 1 |
| Central nervous system syphilis | 1 |

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## Clinical Results

*Pneumonia.* Of the 55 cases of pneumonia treated in the hospital, all but two were improved or cured. The pneumococcus was the etiologic agent in 38 cases, *Streptococcus* in 12, *Staphylococcus aureus* in 4, and an undetermined agent in 1. The two treatment failures occurred in the penicillin-sensitive *S. aureus* group. Both of these patients subsequently responded to high-dose intravenous penicillin G therapy. The 38 cases of pneumococcal pneumonia were most often treated with 500 mg of phenethicillin (range: 250 to 1,500 mg) every 6 hr. Therapy was continued in most patients for 7 to 13 days (range: 1 to 21 days). Clinical response was observed in 26 of the 38 cases within the first 24 hr; the other 12 responded within 96 hr. There is no doubt that under suitable circumstances, where oral medication can be tolerated, phenethicillin therapy is effective in the control of pneumococcal pneumonic infections.

*Tonsillitis.* The 51 patients with $\beta$-hemolytic streptococcal tonsillitis and pharyngitis received 250 mg of phenethicillin three or four times daily for 10 to 18 days. The bacterial cure rate was 89%.

*Subacute bacterial endocarditis.* The 18 patients with subacute bacterial endocarditis received phenethicillin for 4 to 6 weeks. The three cases treated despite negative blood cultures will be omitted from this discussion, thus leaving 15 cases with positive blood cultures. The etiologic agent was $\alpha$-hemolytic streptococcus in 11 cases, penicillin-sensitive *S. aureus* in 2 cases, and gamma and anaerobic streptococcus in 1 case each. Therapy consisted of 24 to 36 tablets of phenethicillin daily, with the majority of patients receiving 1,000 to 1,500 mg (4 to 6 tablets) every 4 hr. (Streptomycin, 1 g daily, was also administered to eight patients for a 2-week period.) Four patients experienced mild gastrointestinal distress manifested by abdominal cramps and loose nondiarrheal stools. In no instance was it necessary to discontinue phenethicillin. There were 14 bacteriological and 13 clinical cures during the immediate treatment period. One patient developed congestive heart failure and another a cerebral embolus while under therapy. There was one death (the patient treated for an anaerobic streptococcal endocarditis, 3 months after therapy was completed).

The last six cases of $\alpha$-hemolytic streptococcal endocarditis have been managed in the following manner. As soon as the bacteriological diagnosis was confirmed, the patient received intravenous aqueous penicillin G, 12 million to 24 million units daily. After 7 days, oral phenethicillin, in the doses previously described, was substituted for the intravenous penicillin. Serum inhibition studies performed on each regimen (penicillin G and phenethicillin) most often revealed similar bactericidal effects in the 1:32 to 1:128 range. All patients received additional streptomycin, 1 g daily for 2 weeks.

The six patients were discharged from the hospital after 14 days and continued their bed rest and oral medication at home, under the supervision of their physician, for an additional 14 days. It must be noted that these cases were highly selected. In each case the streptococcus was markedly sensitive to penicillin, and there was no evidence of embolization or congestive heart failure. A follow-up of 6 to 18 months has revealed no relapses or serious complications in this group.

*L-Phenethicillin.* Levopotassium phenoxyethyl penicillin, the L isomer or phenethicillin, has been reported to exhibit greater biological activity than the racemic mixture, DL-alphaphenoxyethyl penicillin. This was particularly true for *S. aureus* strains (McCarthy, Wallmark, and Finland, 1961).

Fifty patients were treated with L-phenethicillin (250 mg tablet three times per day). In this group of patients were 20 with acute and chronic bronchitis, 14 with tonsillitis, 9 with skin and soft-tissue infections, and 7 with pneumonia. Results were uniformly satisfactory; most significant was the rapid and favorable response in eight of nine staphylococcal skin and soft-tissue infections.

*Methicillin.* 2,6-Dimethoxyphenyl penicillin (methicillin) was prescribed and evaluated in the management of 50 moderate and severe penicillin-resistant staphylococcal infections. Initial dosage was 1 g, intramuscularly, four to six times per day. However, early in this series it became evident that better results were obtained when the dose was raised to 12 to 16 g daily. The drug has been well tolerated intravenously, as well as intramuscularly in cases receiving as many as 264 injections during a single course of therapy.

The most striking results were observed in the management of severe staphylococcal pneumonia. Twelve patients varying in age from 32 to 80 were treated with methicillin. Eleven of these patients received no other antibiotic. Nine cases were classified as hospital-acquired infections, and severe underlying debilitating disease was present in all but two individuals. A high index of suspicion of staphylococcal pneumonia in this series led us to administer methicillin as the drug of choice 24 to 48 hr before cultures and sensitivity reports were available. Duration of therapy ranged from 10 to 75 days; 10 of the 12 patients survived (Table 2).

We did not fare as well with our seven cases of staphylococcal septicemia (Table 2). Despite bacteriological success, four of seven patients died. Two patients with associated neoplastic disease succumbed to disseminated *Candida* superinfection.

Two cases of resistant staphylococcal endocarditis were treated, with survival in one (Table 2). In both cases, cultures remained positive during the first week on a 6-g daily methicillin schedule, but converted to negative when the daily dose was raised to 16 g. After 6 weeks, both patients were discharged from the hospital, but

one patient returned 1 month later; although blood cultures were negative, the patient died of progressive aortic valvular disease and congestive heart failure.

TABLE 2. *Results of treatment of staphylococcal infections with methicillin*

| Diagnosis | No. of cases | Survived | Died |
|---|---|---|---|
| Pneumonia | 12 | 10 | 2 |
| Septicemia | 7 | 3 | 4 |
| Endocarditis | 2 | 1 | 1 |
| Meningitis | 1 | | 1 |
| Other* | 28 | 27 | 1 |

*Skin and soft-tissue infections, 14; urinary tract infections, 7; osteomyelitis, 4; enterocolitis, 2; and parotitis, 1.

Methicillin therapy proved satisfactory in the management of resistant staphylococcal osteomyelitis, prostatitis and pyelonephritis, parotitis, and skin and soft-tissue infections (Table 2). Side reactions were limited to rash in one case and two instances of drug fever on the high dose (16 g daily) regimen.

## Discussion

The mortality of severe antibiotic-resistant staphylococcal disease in the pre-methicillin era was reported to be between 60 and 80%. It is now possible, with the prompt administration of this drug, to reverse the trend and lower the mortality to 20%. This is true in most infections except septicemia, where host factors are of prime importance.

On the basis of the results reported here, methicillin therapy would appear to be indicated for penicillin-resistant staphylococcal infections, such as: pneumonia, septicemia, endocarditis, meningitis, and osteomyelitis.

Phenethicillin would be indicated for: $\beta$-hemolytic streptococcal tonsillitis, pneumococcal pneumonia, selected cases of $\alpha$-hemolytic streptococcal endocarditis, and skin and soft-tissue infections due to penicillin-sensitive *S. aureus*.

## Literature Cited

McCarthy, C. G., G. Wallmark, and M. Finland. 1961. In vitro activity of various penicillins. Am. J. Med. Sci. 241:143-159.

# COMPARISON OF THREE PENICILLINS AGAINST MICROORGANISMS CAUSING BACTERIAL ENDOCARDITIS WITH SOME CLINICAL OBSERVATIONS ON PHENETHICILLIN

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The treatment of endocarditis with oral penicillin G is not considered reliable because of the variable gastrointestinal destruction and absorption of the compound (Finland, 1954). The development of more acid-stable and better-absorbed natural and semisynthetic penicillins has circumvented this particular criticism. The successful treatment of endocarditis with penicillin V (phenoxymethyl penicillin) has been reported (Quinn et al., 1956; Martin, Nichols, and Heilman, 1956; Santos-Buch, Koenig, and Rogers, 1957; Walker and Hamburger, 1957; Quinn and Colville, 1961). With the development (Batchelor et al., 1959) of a new semisynthetic penicillin, phenethicillin (phenoxyethyl penicillin, later referred to as penicillin B), which produced relatively high serum concentrations of penicillin activity (McCarthy and Finland, 1960; Fairbrother and Taylor, 1960), there seemed to be little reason for not accepting oral administration of penicillin as the routine method for treating endocarditis.

A possible major objection to such a course concerned the question of whether or not these newer penicillins were as effective as penicillin G in their antibacterial activity against the microorganisms causing endocarditis. Some observations based on a comparison in sensitivity tests showed that penicillin G was more effective against streptococci (Garrod, 1960). On that basis, penicillin G and, therefore, parenteral penicillin would continue to offer optimal treatment for penicillin-sensitive strains causing endocarditis. Because sensitivity tests can be shown to give widely different results according to the test conditions used (Jackson and Finland, 1951), it was decided to test penicillin B simultaneously with penicillin V and penicillin G against streptococci causing endocarditis in media of different

composition and to observe end points of antibacterial activity other than the usual minimal concentration that inhibited growth of the bacterial strain.

## Materials and Methods

*Bacterial strains.* The bacterial strains recovered from the blood of patients admitted to the hospital with endocarditis were subpassaged in tryptophosphate broth until good growth was obtained. A total of nine strains are included in this study. Of these, six strains were alpha hemolytic and one was a nonhemolytic streptococcus. All of these were inhibited by less than 0.1 unit of penicillin, with minimal inhibitory concentrations in the range of 0.015 to 0.06. Two strains of enterococcus that had minimal inhibitory concentrations of more than one unit, but less than 10 units, of penicillin also were studied.

*Antibacterial tests.* The three penicillins were adjusted to equal unitage according to their bioassay as follows, in units/mg: penicillin G, 1650; penicillin V, 1530; and penicillin B, 1476. The phenethicillin (penicillin B) used in these studies was kindly furnished by Bristol Laboratories, Syracuse, N.Y. Serial twofold dilutions were made from 250 to 0.12 units/ml. Because protein binding of penicillin can be one of the most important factors in altering the apparent antibacterial activity, all tests were performed simultaneously in duplicate in serum-free medium and in 80% serum broth medium. The parameters devised for comparison of the in vitro antibacterial activity of the three penicillins are shown in Fig. 1.

In addition to the classical observation of bacterial growth and the minimal inhibitory concentration, other end points were devised to test different properties and rates of the penicillin activity. These parameters and their definitions are as follows: (i) the avidity-lysis concentration, the least concentration of penicillin which killed or obscured 99% of the colonies in the lag phase when tested by transfer to antibiotic-free medium with penicillinase within a few minutes after inoculation; (ii) a 6-hr bactericidal concentration, the smallest amount of penicillin required to kill 99.99% of the inoculum after 6 hr of incubation; (iii) the minimal bactericidal concentration, the lowest concentration of penicillin that was bactericidal for approximately 99.99% of the colonies after 24 hr of incubation; (iv) clonal variance, the highest concentration of penicillin in which a small number of colonies, but at least 0.05% of the inoculum, persisted after 24 hr of incubation; and (v) the log-phase killing rate, the log decrease per hour in the number of viable organisms between the third and sixth hours of incubation in the minimal bactericidal concentration of penicillin.

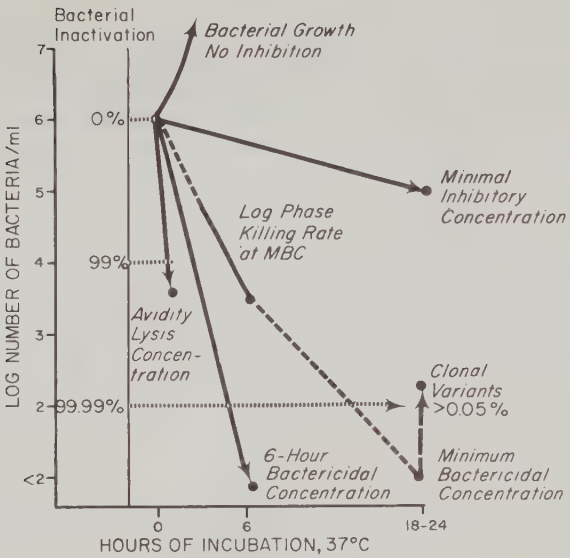


FIG. 1. *Parameters of in vitro antibacterial activity for comparison of penicillins.*

*Antibiotic levels.* The phenethicillin concentration in the serum of patients receiving treatment was determined by serial twofold dilutions using *Staphylococcus aureus* 209 P as the indicator strain. The results were expressed as μg of phenethicillin base per ml of serum.

*Homologous bactericidal tests.* Serum from the patients was collected on different days throughout the treatment regimen. The timed killing effect of the undiluted serum specimen against a heavy inoculum of the homologous strain was measured. Serum dilutions in 6% human albumin also were sometimes measured for timed bactericidal activity.

## Results

The relative bactericidal capacity of the three penicillins, tested in serum-free broth medium against nine bacterial strains of streptococci recovered from patients with endocarditis, is shown according to the bactericidal indices previously defined (Fig. 2). The

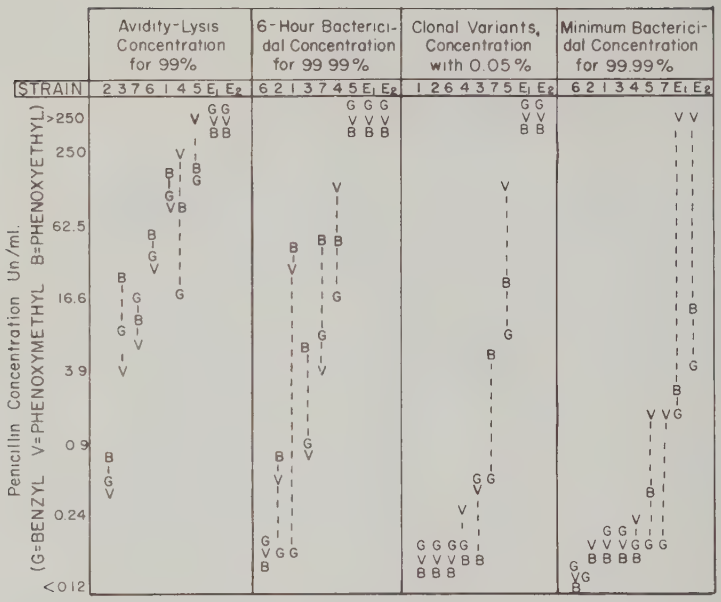


FIG. 2. *Comparison of antistreptococcal effect of penicillins in serum-free medium. This shows the relative activity of penicillins G, V, and B against strains of streptococci recovered from patients with endocarditis, by four parameters of bactericidal action. Strains 1 to 7 were alpha-hemolytic streptococci, except number 6, which was nonhemolytic. Strains $E^1$ and $E^2$ were enterococci.*

avidity-lysis concentration which was presumed to measure the relative binding capacity of the essentially resting bacterial cells for each of the penicillins is shown in the first column. Among the non-enterococcal strains, numbered one to seven, it is apparent that there were very appreciable differences, whereas differences among the penicillins were not greater than fourfold with the exception of one strain. Differences in the 6-hr bactericidal concentration for the different strains varied over the entire test range of a 2048-fold change in penicillin concentration. Penicillin G was nearly always as effective at the same and sometimes at a remarkably lower concentration than the concentrations of penicillins B and V which were required for a 6-hr bactericidal effect. The differences among strains, however, was again the major finding. Clonal variants in excess of 0.05% of the inoculum from two of the strains of alpha streptococci persisted in rather high concentrations of penicillin. In these few instances, benzyl penicillin was effective in eliminating persistors at concentrations 2 to 16 times lower than penicillins V or B. After 24 hr of incubation, the bactericidal concentration of

penicillin G for all of the nonenterococci strains was less than one unit and not significantly different for penicillins V and B. It was only in this last parameter that any difference was observed among the penicillins for the two strains of enterococcus. The results showed penicillins G and B to be bactericidal at significantly lower concentrations than penicillin V.

The results were somewhat altered by the tests in 80% serum-broth (Table 1). The mode effect on the measurements that were made was nil or within the experimental range for all of the penicillins. Large shifts did occur, however, in certain tests. The bactericidal activity of penicillin G at either 6 or 24 hr was decreased by serum proteins if any change was observed. The major direction of the shift in bactericidal activity of penicillin B, induced by serum protein, was also toward the requirement of a higher concentration for some strains. In contrast to penicillins G and B, the direction of the major shift in the activity of penicillin V in serum was toward an increased bactericidal effect. The data suggest that the protein binding of penicillins G and B is greater than for penicillin V.

TABLE 1. *Effect of high serum concentration (80%) on the antibacterial activity of penicillins against strains of alpha streptococcus*

| | Fold change from result in serum-free broth | | | | | |
|---|---|---|---|---|---|---|
| | Penicillin G | | Penicillin V | | Penicillin B | |
| Measurement | Range | Mode | Range | Mode | Range | Mode |
| Avidity-lysis concentration | −4 to +2 | −4 | −2 to 0 | 0 | −4 to 0 | 0 |
| 6-Hr bactericidal concentration | 0 to +8 | 0 | −16 to +4 | 0 | −4 to +16 | −2 |
| Clonal variants concentration | −2 to 0 | 0 | 0 to +2 | 0 | −2 to +4 | −2 |
| Minimal bactericidal concentration | 0 to +8 | 0 | 16 to +4 | 0 | −4 to +16 | 0 |

In addition to penicillin binding, the effect of serum on the growth rate and survival of alpha streptococci affected the results in two ways as shown for two different inocula (Fig. 3). Serum accelerated the early phase growth rate of the small inoculum and encouraged the persistence of viable cells. The accelerated growth rate induced by serum may overcome its penicillin-binding effect and account for the mode difference in antibacterial effect. In the presence of penicillin, serum did not appreciably increase the number of organisms persisting over that observed in serum-free broth, despite the spontaneous lysis observed in the latter medium in the absence of penicillin.

The time function of the bactericidal activity of penicillins at the minimal bactericidal concentrations during a uniform period of

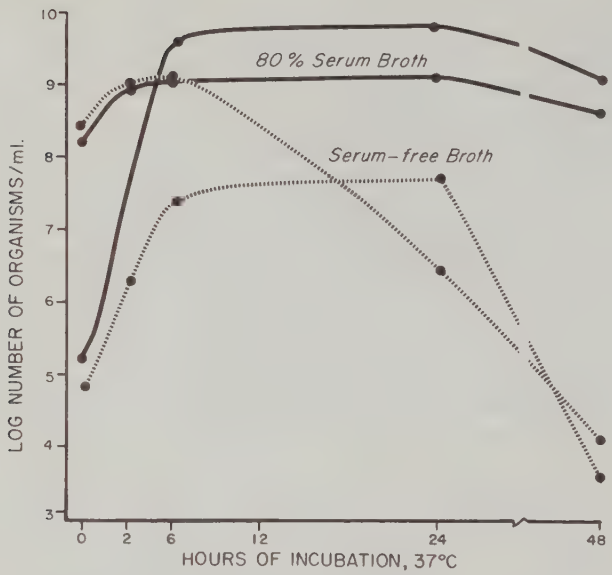


FIG. 3. *Effects of inoculum size and serum upon the growth characteristics of alpha streptococcus strains.*

accelerated bacterial growth is compared for the three penicillins studied (Table 2). These data showed no significant difference in the mean log-phase killing rate of alpha streptococci in broth or 80% serum broth for penicillins G, V, and B. The mean decrease was 0.9 to 1 log per hr. The log-phase killing rate of enterococci by penicillins G and B was only one-third that observed for alpha streptococci and could not be calculated for penicillin V, since the minimal bactericidal concentration exceeded the highest test concentration of penicillin.

TABLE 2. *Mean log phase killing rate* by penicillins*

| Species | Medium | Penicillin G | Penicillin V | Penicillin B |
|---|---|---|---|---|
| Alpha streptococcus | Broth | 0.9 | 1.0 | 1.0 |
| | 80% Serum broth | 0.9 | 0.9 | 0.9 |
| Enterococcus | Either | 0.3 | 0.0 | 0.3 |

*Log decrease in viable organisms per hr between the 3rd and 6th hr of incubation.

The details of treatment of the nine patients from whom the above study strains were obtained will be reported elsewhere. Oral phenethicillin produced a minimal serum concentration of at least 1.0 μg/ml when a dose of 1 g was given every 4 hr or 500 mg every 4 hr with 2 g of probenecid per day. These levels exceeded the minimal observed bactericidal concentration for alpha streptococci by more than 10 times. Serum collected from the patients was studied for homologous bactericidal activity. The strain differences noted by the previous bactericidal indices also were observed to influence the rate of killing by homologous serum. In addition, the time required for killing was inversely related to the log number of organisms inoculated and to the phenethicillin concentration in the homologous serum. In accordance with the in vitro studies, good clinical responses were observed in the patients treated with oral phenethicillin, and all appear to be cured 1 to 19 months after treatment.

## Discussion

Penicillin B is relatively resistant to destruction by acid gastric secretion and is well absorbed from the gastrointestinal tract (McCarthy and Finland, 1960; Fairbrother, 1960). The present studies were designed to determine whether this compound, on a unit to unit basis, was as effective in its antibacterial activity against penicillin-sensitive strains of streptococci causing endocarditis as were penicillin G and a natural, acid-resistant penicillin, penicillin V. Among the questions to be asked in this regard were: (i) Does serum protein inhibit the activity of one or another of the penicillins to a significantly greater degree? Hence, the tests were done in the presence of high serum protein concentrations as well as serum-free broth. (ii) Could a different affinity of the bacterial cell wall be shown for the different penicillins? The avidity-lysis test was used as a measure of irreversible binding of penicillin during the bacterial lag phase. (iii) Since bactericidal activity, not merely inhibition of growth, is the property of major importance in the treatment of endocarditis, are there significant differences in the minimal concentration required to produce nearly complete killing at an early period (6 hr) and a later period (24 hr)? (iv) Does one penicillin permit clonal variants to persist in concentrations of penicillin above the inhibitory and bactericidal concentrations more than another? And finally, (v) is the rate of bacterial killing, during a time when the cells are most susceptible to penicillin due to their vigorous proliferative activity, different for the different penicillin forms under study?

A high concentration of serum proteins in the test medium introduced factors that were both favorable and unfavorable for drug bactericidal activity. The wide differences that sometimes can be

observed in different media for particular strains are a resolution of these divergent forces on the growth of the strain and the binding of penicillin. Often they neutralize one another with a resultant insignificant effect. Thus, 10% serum was found to be more inhibitory for penicillin B than it was for penicillins G or V, whereas 100% serum was less inhibitory (Gourevitch, Hunt, and Lein, 1960). In either medium, comparable antibacterial activity sometimes required differences as great as 256-fold in the amount of one or another of the penicillins; however, the differences among the antibiotics were usually more modest in the range of 4- to 8-fold. Penicillin G tended to show the best activity, penicillin V the least activity, and penicillin B intermediate activity by the parameters measured, but no one penicillin was consistently the best for all strains in all of the tests used. The answer to each of the questions above, therefore, can be given that the three penicillins are quite similar in these properties on a unit basis of comparison.

More striking than the differences among the penicillins in their antibacterial activity for a particular strain was the observed difference among "penicillin-sensitive" strains when examined by the new parameters. Large differences were observed in the strain affinity for penicillin, the early bactericidal concentrations, the clonal variance, and the rate of bacterial killing by penicillin. Some strains of alpha-hemolytic streptococcus approached the behavior of enterococci in their poor "binding" of penicillin and the slow rate of bacterial death, whereas others were killed rapidly by small concentrations of penicillins and left few or no persistors. These important differences among strains are not well detected by the usual sensitivity tests and may be of value in prescribing the dose and the duration of penicillin treatment. For this purpose the 6-hr bactericidal concentration was the most discriminating, and its use is recommended in the study of strains causing endocarditis as a more meaningful index than the minimal inhibitory concentration.

The experience from these studies suggests that phenethicillin can be considered equally as effective as benzyl penicillin or penicillin V in the eradication of streptococci causing endocarditis.

The clinical observations also indicate that endocarditis caused by penicillin-sensitive strains can be treated optimally by oral phenethicillin. The homologous serum bactericidal activity was greatest when the dose given was sufficient to produce serum concentrations equivalent to the 6-hr bactericidal concentration. Successful, but perhaps less than optimal, treatment can be achieved with lower serum concentrations above the minimal bactericidal concentration.

## Literature Cited

Batchelor, F. R., F. P. Doyle, J. H. Naylor, and G. N. Robinson. 1959. Synthesis of penicillin: 6-aminopenicillanic acid in penicillin fermentations. Nature **183**:257-258.

Fairbrother, R. W., and G. Taylor. 1960. Observations of penicillin "B". Lancet **2**:400-402.

Finland, M. 1954. Treatment of bacterial endocarditis. New Engl. J. Med. **250**:372-383, 419-428.

Garrod, L. P. 1960. Relative antibacterial activity of three penicillins. Brit. Med. J. **1**:527-529.

Gourevitch, A., G. A. Hunt, and J. Lein. 1960. Microbiological studies on potassium penicillin-152 (potassium (alpha-phenoxyethyl)-penicillin). Antibiotics Ann. 1959-1960, p. 111-118.

Jackson, G. G., and M. Finland. 1951. Comparison of methods for determining sensitivity of bacteria to antibiotics *in vitro*. A. M. A. Arch. Internal Med. **88**:446-460.

Martin, W. J., D. R. Nichols, and F. R. Heilman. 1956. Observations on clinical use of phenoxymethyl penicillin (penicillin V). J. Am. Med. Assoc. **160**:928-931.

McCarthy, C. G., and M. Finland. 1960. Absorption and excretion of four penicillins: penicillin G, penicillin V, phenethicillin and phenylmercaptomethyl penicillin. New Engl. J. Med. **263**: 315-326.

Quinn, E. L., J. M. Colville, F. Cox, Jr., and J. Truant. 1956. Phenoxymethyl penicillin (penicillin V) therapy of subacute bacterial endocarditis. J. Am. Med. Assoc. **160**:931-936.

Quinn, E. L., and J. M. Colville. 1961. Subacute bacterial endocarditis. Clinical and laboratory observations in 27 consecutive cases treated with penicillin V by mouth. New Engl. J. Med. **264**:835-842.

Santos-Buch, C. A., M. H. Koenig, and D. E. Rogers. 1957. Oral treatment of subacute bacterial endocarditis with phenoxymethyl penicillin (penicillin V). New Engl. J. Med. **257**:249-257.

Walker, W. F., and M. Hamburger. 1957. Penicillin-sensitive streptococcal endocarditis: report of cases treated for two weeks with oral phenoxymethyl penicillin and intramuscular streptomycin. A. M. A. Arch. Internal Med. **100**:359-363.

# POTASSIUM PHENETHICILLIN AND POTASSIUM PENICILLIN V IN THE TREATMENT OF GROUP A STREPTOCOCCAL PHARYNGITIS

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The introduction in 1959 of potassium phenethicillin (K phenethicillin), the first semisynthetic penicillin (Perron et al., 1960), was followed by considerable interest in its special biological properties. The chemical structure of K phenethicillin differs from that of K penicillin V by substitution of a methyl radical for a methyl hydrogen on the phenoxy methylene group. The newer penicillin contains a mixture of D- and L-isomers. It resembles K penecillin V in its stability in acid media (Gourevitch, Hunt, and Lein, 1960).

K phenethicillin has been reported to produce higher blood levels and urinary excretion than K penicillin V, after ingestion of equivalent dosage (Morigi, Wheatley, and Albright, 1960; Martin and Nichols, 1960). These higher concentrations, however, have not been accompanied by greater antistreptococcal activity in the serum (McCarthy and Finland, 1960; Griffith, 1960). The significance of these in vitro findings has not been clear.

This study was designed to compare the effectiveness against streptococcal pharyngitis of K phenethicillin and K penicillin V, administered orally in a dose of 750 mg (1,200,000 units) daily. K penicillin V has been demonstrated to be effective in eradicating beta-hemolytic streptococci from the pharynx (Breese and Disney, 1958; Stillerman et al., 1960).

## Materials and Methods

The subjects were 137 private patients of two pediatricians, practicing among families of midsocio-economic level in a Long Island suburb of New York City.

The throats of patients with acute pharyngitis, suspected of being streptococcal, were cultured from December 1959 to June 1960. The swabs were smeared directly onto 10% sheep blood agar plates which were streaked and incubated for 18 to 48 hr. The diagnosis of

streptococcal pharyngitis was based upon the growth of beta-hemolytic streptococci on the culture plates. From February to June, 1960, one of the authors (M. S.) also inoculated one of the throat swabs into a tube of broth for determination of penicillin-inhibiting microbial end products.

Alternate patients, severely ill at the first visit, were treated orally with K phenethicillin (Bristol Laboratories, Syracuse, N.Y.) or K penicillin V (Eli Lilly & Co., Indianapolis, Ind.), 250 mg, on arising, at 2:00 PM, and at bedtime without awaiting culture results. When the culture was positive the treatment was continued for 10 full days and, when negative, was continued as circumstances indicated.

Alternate patients, mildly or moderately ill, on positive culture determination were treated with K phenethicillin or K penicillin V in the same dosage schedule.

Follow-up cultures were taken frequently during treatment and up to a limit of 35 days thereafter. In the two treatment groups, 60% of the cases had three or more cultures after treatment was completed.

The effectiveness of therapy was determined by the complete or incomplete eradication of hemolytic streptococci from the pharynx. The results of treatment were listed as cure, failure, relapse, or reinfection. "Cure" refers to cases in which cultures were negative after the fifth day of treatment, and one or more cultures were negative 8 to 35 days after completing 10 days of therapy. "Failure" refers to persistence of streptococci during the second 5 days of treatment. "Relapse" indicates persistence or recurrence of hemolytic streptococci of the same group and type as the pretreatment culture, 1 to 35 days after cessation of treatment. "Reinfection" designates recurrence of streptococci of a different group or type, 1 to 35 days after end of treatment. The reinfections were considered cures if at least one negative culture, obtained 8 or more days after the end of treatment, preceded the reinfection. Recurrence of hemolytic streptococci which could not be recovered on subculture were noted as "unknowns".

An adjusted cure rate was used for analysis, since "reinfection" usually indicates the acquisition of a new streptococcal infection. Only reinfections which met the criteria for "cures" were included in our tabulations. Five reinfections after K phenethicillin and three after K penicillin V were deleted either because they occurred 1 to 7 days after the end of treatment, or because they were not preceded by a negative culture at least 8 days after the end of treatment. This adjusted cure rate was computed by adding to the number of cured cases the number of reinfections considered as cures and the prorated number of recurrences with "unknowns". The prorated number of "unknowns" presumed to be reinfections was obtained by applying to the total number of unknowns a ratio of the reinfections to the reinfections plus relapses.

Bacterial recurrence detected 1 to 35 days after end of therapy were treated again, orally with propionyl erythromycin (Eli Lilly & Co., Indianapolis, Ind.), 30 mg per kg per day, divided into three doses daily for 10 days.

Whenever recurrences developed, household contacts were cultured for possible sources for reinfection.

The serologic group and type of one of the surface colonies of hemolytic streptococci were determined by the method of Swift, Wilson, and Lancefield (1943). Streptococcal antisera for groups A, B, C, D, F, and G and group A types 1 to 6, 11 to 15, 17 to 19, 22 to 24, 26 to 33, 36 to 44, and 46 to 48 were obtained from the Communicable Disease Center, Atlanta, Ga. Only cases categorized as group A were evaluated in this study. Nine strains of group A streptococci, isolated from cases which relapsed, were tested for sensitivity to K phenethicillin and K penicillin V by the tube-dilution method. Broth cultures of the pharyngial flora obtained from patients before, during, and after completion of treatment were tested for penicillin-inhibitory activity by a method devised by Bernstein, Stillerman, and Samet (1960).

## Results

*Response during therapy.* Clinical improvement, as manifested by a return of temperature to normal in 24 to 48 hr and rapid lessening of the sore throat, occurred equally in practically all cases of both groups. During the first 5 days of therapy, beta-hemolytic streptococci were recovered on the third day of therapy from one of 21 patients treated with K phenethicillin and from none of 18 patients treated with K penicillin V. During the second 5 days of therapy, no positive cultures, indicating "failures", were obtained in 45 cases treated with K phenethicillin or in 38 cases treated with K penicillin V.

*Bacterial outcome 1 to 35 days after end of therapy.* The proportion of bacterial cures and recurrences among the 137 cases treated with K phenethicillin and K penicillin V is given in Fig. 1. The adjusted cure rates, hereafter referred to as cure rates, were 85% among 69 cases treated with K phenethicillin and 91% among 68 cases treated with K penicillin V. The difference in cure rates is not statistically significant ($p > 0.10$).

*Cure rates for selected characteristics.* Comparison of the number of cases in each treatment group, according to specific characteristics, shows no significant differences between the two treatment groups (Table 1).

Analysis of the cure rates for the specific characteristics, within each group as well as between the two groups, indicated no significant differences. Despite the fact that the cure rates for K

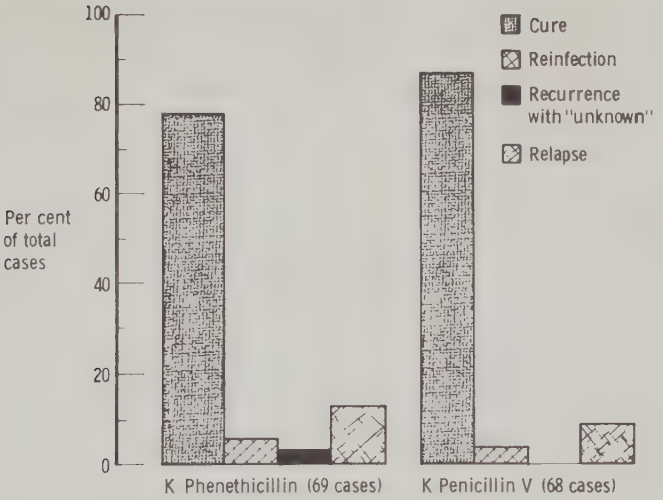


FIG. 1. *Bacterial outcome of treatment of group A streptococcal pharyngitis.*

TABLE 1. *Adjusted cure rates for specific characteristics in two treatment groups*

| Characteristic | K Phenethicillin (69 cases) | | K Penicillin V (68 cases) | |
|---|---|---|---|---|
| | No. of cases | Cured | No. of cases | Cured |
| | | % | | % |
| New infection | 66 | 86 | 65 | 91 |
| Carrier state | 3 | * | 3 | * |
| December to February | 39 | 77 | 31 | 87 |
| March to June | 30 | 93 | 37 | 94 |
| Under 5 years | 7 | 86 | 5 | * |
| 5 to 10 years | 49 | 80 | 54 | 91 |
| 11 to 15 years | 13 | 100 | 9 | * |
| Male | 37 | 97 | 34 | 91 |
| Female | 32 | 88 | 34 | 91 |
| Tonsils in | 66 | 83 | 66 | 92 |
| Tonsils out | 3 | * | 2 | * |
| Day of illness treated | | | | |
| 1 to 3 | 52 | 82 | 53 | 90 |
| 4 and over | 17 | 82 | 13 | 86 |
| Pretreatment cultures | | | | |
| 1+ to 2+ | 17 | 88 | 12 | 83 |
| 3+ to 4+ | 52 | 83 | 56 | 93 |
| Group A strains | | | | |
| Typable | 43 | 81 | 50 | 90 |
| Nontypable | 26 | 88 | 18 | 94 |

*N too small for valid t evaluation.

phenethicillin and K penicillin V do not differ significantly for the individual characteristics analyzed, K penicillin V showed a higher cure rate in 11 of the 13 comparisons indicated.

*Relapse intervals and retreatment with erythromycin.* Among 10 patients with bacterial relapses following K phenethicillin, seven also had clinical manifestations which developed 4 to 31 days (average: 14 days) after the end of treatment. Retreatment of seven of the 10 relapses with propionyl erythromycin was followed by cure in all patients treated again.

Among six patients with bacterial relapse following K penicillin V, four had clinical manifestations which developed 4 to 9 days (average: 7 days) after the end of treatment. When five of the six relapses were treated again with propionyl erythromycin, cures resulted in two of the five patients.

*Penicillin inhibition by pharyngeal flora.* Bacterial end products in pharyngeal cultures were found to inhibit penicillin (0.1 $\mu$g/ml) in 18% of 85 nonstreptococcal cases and 22% of 74 streptococcal cases. Among the 74 streptococcal cases, penicillin inhibition was noted in 10 of 63 (16%) cured cases and six of 11 relapses. Of the six relapses, four occurred in the K phenethicillin group and two in the K penicillin V group. When treated again with erythromycin, five of the six relapses showing penicillin inhibition resulted in cures. Use of erythromycin in four of five relapses showing no penicillin inhibition yielded cures in two cases. The organism most frequently isolated from broth cultures exhibiting penicillin-inhibition, was the hemolytic, coagulase-positive *Staphylococcus aureus*.

*Minimal inhibitory concentrations.* Table 2 shows the minimal inhibitory concentrations for K phenethicillin to be four times higher than that for K penicillin V against eight of nine strains of group A streptococci.

TABLE 2. *Minimal inhibitory concentrations of K phenethicillin and K penicillin V against group A streptococci*

| MIC | K Phenethicillin No. of strains | K Penicillin V No. of strains |
|---|---|---|
| $\mu g/ml$ | | |
| 0.008 | | 8 |
| 0.012 | | 1 |
| 0.016 | 1 | |
| 0.03 | 8 | |

*Cultures from household contacts.* Among the cases with relapses, household contacts, harboring streptococci of the same group and type as the original case, were revealed as possible sources of reinfection in four of 10 cases treated with K phenethicillin and four of six cases treated with K penicillin V.

*Adverse effects.* The penicillin preparations were well tolerated. Potassium phenethicillin was discontinued in one allergic patient with a history of sensitivity to penicillin, owing to urticaria, on the second day of administration.

No suppurative or nonsuppurative complications were observed.

## Discussion

With similar treatment groups and with bacteriological findings as the criterion of successful treatment, K phenethicillin and K penicillin V were found to be about equally effective in the treatment of streptococcal pharyngitis. The trend for a higher cure rate favored K penicillin V.

The penicillin dose of 750 mg daily was selected because our over-all experience from 1956 to 1959 indicated that potassium penicillin V was 11% more effective in a dose of 750 mg than 375 mg daily, yielding 89 and 78% cure rates, respectively (Stillerman and Bernstein, *to be published*). The cure rate with K penicillin V in the present series is the same as that obtained in the 1956 to 1959 series with the 750 mg daily dose.

Clinical manifestations were noted in 11 of 16 bacterial relapses. In nine of the 11 cases, the clinical manifestations developed within 14 days after ending treatment. This agrees with our previous findings (Stillerman et al., 1960) and suggests that the recommended follow-up culture time of 2 days (American Heart Association. Committee on Prevention of Rheumatic Fever and Bacterial Endocarditis, 1960) be extended to at least 2 weeks. The remaining five asymptomatic bacterial relapses would have been missed if follow-up cultures had not been taken routinely.

The greater antistreptococcal activity of K penicillin V than K phenethicillin in vitro agrees with that reported by Garrod (1960).

The presence of penicillin inhibition in a significantly higher proportion of cases with relapse than cases with cure suggests this as a possible cause of relapse. However, the number of relapses showing penicillin inhibition in each treatment group is too small to warrant statistical evaluation.

Although household contacts, harboring the same serologic streptococci as those recovered from patients with relapses, are known to be a source of reinfection, their significance could not be adequately evaluated owing to the small number of cases in each category.

This study indicates that the first semisynthesized penicillin offers no advantage over K penicillin V in the treatment of streptococcal pharyngitis.

## Acknowledgments

The technical assistance of Lucia Tomkus and Lois Mishcon and statistical assistance by Vincent Fisichelli are gratefully acknowledged.

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We are indebted to R. S. Griffith of the Lilly Research Laboratories for streptococcal sensitivity testing by the tube-dilution method.

## Literature Cited

American Heart Association. Committee on Prevention of Rheumatic Fever and Bacterial Endocarditis. 1960. Prevention of rheumatic fever and bacterial endocarditis through the control of streptococcal infection. Circulation **21**:151-155.

Bernstein, S. H., M. Stillerman, and C. M. Samet. 1960. Demonstration of penicillin inhibition by pharyngeal flora in patients treated for streptococcal pharyngitis. Circulation **22**:724.

Breese, B. B., and F. A. Disney. 1958. Penicillin in the treatment of streptococcal infections; a comparison of effectiveness of five different oral and one parenteral form. New Engl. J. Med. **259**:57-62.

Garrod, L. P. 1960. Relative antibacterial activity of three penicillins. Brit. Med. J. **1**:527-529.

Gourevitch, G. A., A. Hunt, and J. Lein. 1960. Microbiological studies on potassium penicillin-152 [potassium (α-phenoxyethyl)-penicillin]. Antibiotics Ann. 1959-1960, p. 111-118.

Griffith, R. S. 1960. Comparison of antibiotic activity in sera after the administration of three different penicillins. Antibiotic Med. & Clin. Therapy **7**:129-135.

McCarthy, C. G., and M. Finland. 1960. Absorption and excretion of four penicillins. New Engl. J. Med. **263**:315-326.

Martin, W. J., and D. R. Nichols. 1960. Penicillin V and phenethicillin potassium in serum: comparison of concentrations and of antibacterial effects. Proc. Staff Meetings Mayo Clin. **35**: 577-584.

Morigi, E. M. E., W. B. Wheatley, and H. Albright. 1960. Clinical and laboratory studies with potassium penicillin-152 [potassium(α-phenoxyethyl) -penicillin]: a new synthetic penicillin. Antibiotics Ann. 1959-1960, p. 127-132.

Perron, Y. G., L. B. Crast, W. J. Gottstein, W. F. Minor, and L. C. Cheney. 1960. Synthesis of potassium penicillin-152 [potassium(α-phenoxyethyl)-penicillin] and both diastereoisomers. Antibiotics Ann. 1959-1960, p. 107-110.

Stillerman, M., S. H. Bernstein, M. L. Smith, S. B. Gittelson, and S. Karelitz. 1960. Antibiotics in the treatment of beta-hemolytic streptococcal pharyngitis: factors influencing the results. Pediatrics **25**:27-34.

Swift, H. F., A. T. Wilson, and R. C. Lancefield. 1943. Typing group A hemolytic streptococci by M precipitin reactions in capillary pipettes. J. Exptl. Med. **78**:127-133.

# LABORATORY EVALUATION OF ALPHA-PHENOXYPROPYL PENICILLIN

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A new penicillin, alpha-phenoxypropyl penicillin, has been described by Williamson, Morrison, and Stevens (1961). Early studies indicated that there were differences in the diastereoisomers of this penicillin. We have compared the in vitro activity and serum concentrations of levo-alpha-phenoxypropyl penicillin (levopropylcillin) with phenoxymethyl penicillin (penicillin V), phenoxyethyl penicillin (phenethicillin), dimethoxyphenyl penicillin (methicillin), and alpha-aminophenyl penicillin. The structural relationship of these five penicillins is shown in Fig. 1.

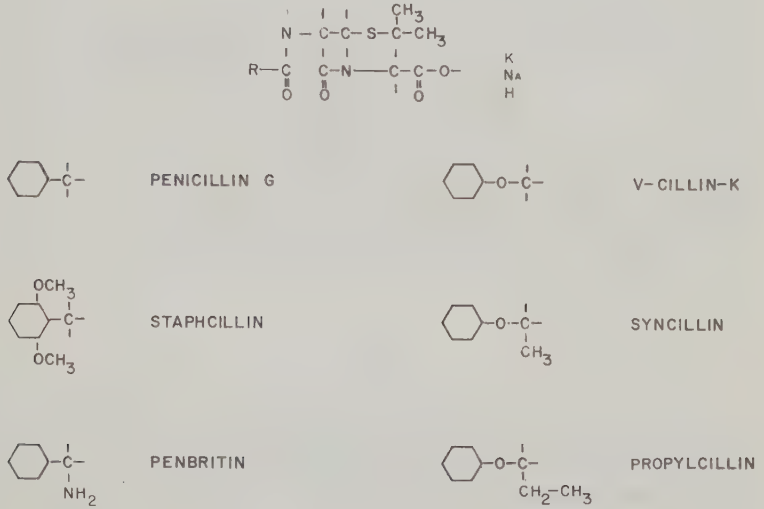


FIG. 1. *Structural relationship of penicillin G, methicillin (Staphcillin), alpha-aminophenyl penicillin (Penbritin), penicillin V (V-Cillin K), phenethicillin (Syncillin), and levopropylcillin (Propylcillin).*

## Materials and Methods

*Sensitivity studies.* All bacterial strains studied were patient-isolates, with the exception of two laboratory strains, beta-hemolytic streptococcus C203 and coagulase-positive *Staphylococcus aureus* 209P.

The serial twofold tube dilution technique was utilized for all sensitivity tests (Kolmer, 1945). Brain heart infusion broth was used as the medium and for preparing all dilutions, unless otherwise indicated. The standard inoculum used was 0.04 ml of a 1:100 dilution of a rapidly growing 18-hr culture of beta-hemolytic streptococcus or of a 1:1000 dilution of coagulase-positive staphylococcus instilled into each tube prior to incubation.

*Blood levels and serum antibacterial activity.* Healthy ambulatory adults were given each test antibiotic orally, with the exception of methicillin which was given intramuscularly. Typical crossover methods were utilized for comparative studies. The subjects were not permitted to smoke or eat from midnight before the test until after the 2-hr blood sample was obtained.

The standard breakfast for those taking the medication with meals was as follows: 570 cal (11 g protein, 63 g carbohydrate, 30 g fat), consisting of 100 ml of orange juice, 20 g of cereal with 60 g of cream, one fried egg, one slice of toast with 10 g of butter, and coffee.

Blood samples were drawn before and at $\frac{1}{2}$, 1, 2, 4, and 6 hr after ingesting the antibiotic under study. These samples were centrifuged and the sera assayed the same day they were drawn. A modification of the Rammelkamp (1942) serial twofold tube dilution technique was used to determine the number of times each sera could be diluted and still retain its antibacterial activity against the test strain of organism. In some instances, the sera samples were divided so that both antibacterial activity and serum concentration could be determined. The *Sarcina lutea* cup-plate method was incorporated for the latter studies. The antibiotic "standard curves" were always made using human serum.

## Results

*Comparative activity of dextro- and levo-alpha-phenoxypropyl penicillin.* Standard metal cups containing 0.4 and 0.8 $\mu$g/ml of the dextro and levo forms of alpha-phenoxypropyl penicillin were placed on agar seeded with *S. lutea* and incubated overnight. The zone size or diameter of inhibition was measured for each preparation. The levo form was more active than the dextro form of this penicillin (Fig. 2).

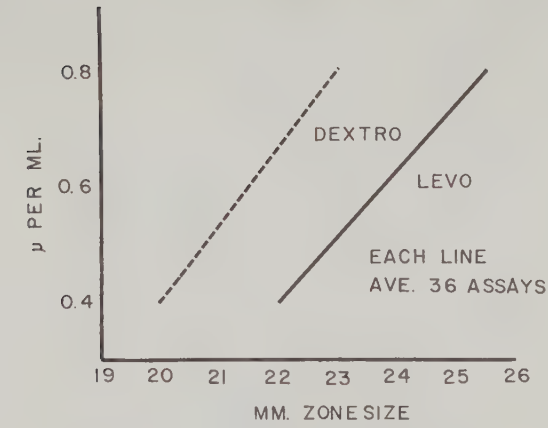


FIG. 2. *Comparison of activity of dextro- and levo-alpha-phenoxypropyl penicillin on Sarcina lutea.*

A single 500-mg dose of dextro- and levo-phenoxypropyl penicillin was given in the typical crossover method to ten fasting subjects. The antibacterial activity in the serum (number of times the serum can be diluted and still inhibit the test organism) of those subjects ingesting the levo form was two to four times that following the dextro-rotatory form (Fig. 3).

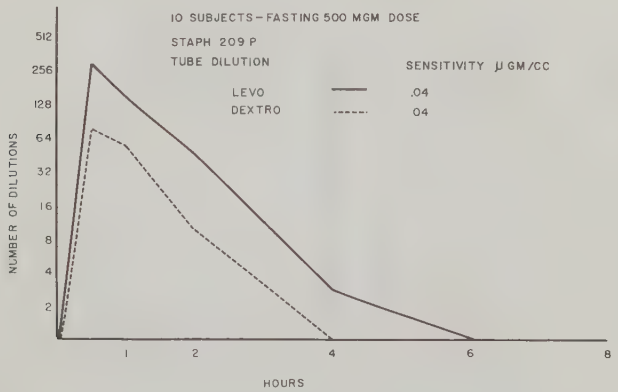


FIG. 3. *Comparison of activity of dextro- and levo-alpha-phenoxypropyl penicillin in serum against Staphylococcus aureus 209 P.*

The antibacterial activity of the sera from the above subjects was also tested against two strains of penicillinase-producing coagulase-positive staphylococci. The greater activity of the levo form against these strains is shown in Fig. 4.

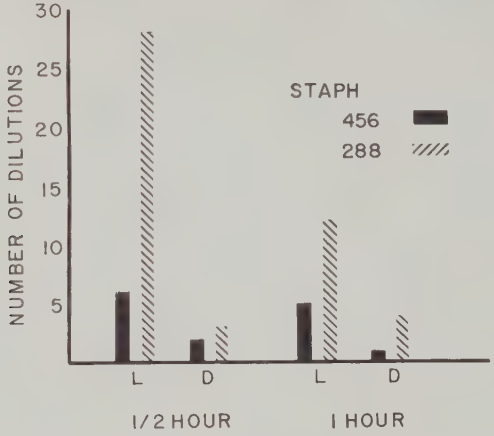


FIG. 4. *Antibacterial activity of sera containing dextro- and levo-alpha-phenoxypropyl penicillin against strains of penicillinase-producing coagulase-positive staphylococci.*

Approximately 410 mg of the levo form was excreted in the urine in the first 6 hr, compared to only 210 mg of the dextro-alpha-phenoxypropyl penicillin (Fig. 5).

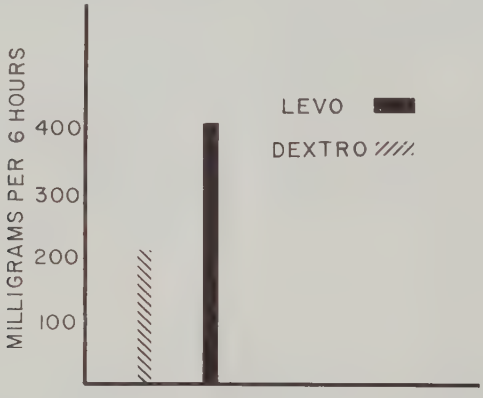


FIG. 5. *Urinary excretion of dextro- and levo-alpha-phenoxypropyl penicillins.*

*In vitro sensitivity.* Beta-hemolytic streptococci require an average of 0.004 $\mu$g of penicillin V per ml for inhibition, compared to 0.012 and 0.014 $\mu$g of phenethicillin and levopropylcillin, respectively.

These same organisms are susceptible to approximately 0.19 μg of methicillin (Table 1).

TABLE 1. *In vitro sensitivity of beta-hemolytic streptococci to penicillins**

| Strain | Pen V | Phen. | Prop. | Meth. |
|---|---|---|---|---|
| C203 | .003 | .003 | .003 | .10 |
| HB51 | .003 | .006 | .012 | .10 |
| HB60 | .003 | .003 | .004 | .05 |
| HB61 | .003 | .012 | .003 | .10 |
| HB74 | .003 | .012 | .003 | .10 |
| HB83 | .003 | .003 | .012 | .10 |
| HB84 | .003 | .05 | .05 | .78 |
| HB86 | .003 | .006 | .012 | .10 |
| HB48 | .004 | .003 | .006 | .19 |
| HB50 | .004 | .006 | .037 | .29 |
| HB58 | .006 | .025 | .012 | .10 |
| Avg. | .004 | .012 | .014 | .19 |

*Pen V = phenoxymethyl penicillin; Phen. = phenethicillin; Prop. = levo-propylcillin; Meth. = methicillin. Results are expressed as minimal inhibitory concentrations (μg/ml).

These studies were repeated with ten additional strains of streptococci and with alpha-aminophenyl penicillin. As indicated by the standard strain of streptococcus (C203), the relative over-all sensitivity of the test method was decreased when compared to the previous study. Alpha-aminophenyl penicillin is almost as active as penicillin V against the strains of beta-hemolytic streptococci studied (Table 2).

TABLE 2. *In vitro sensitivity of beta-hemolytic streptococci to penicillins**

| Strain | Pen V | Phen. | Prop. | Amino. |
|---|---|---|---|---|
| C203 | .008 | .031 | .031 | .016 |
| 15704 | .008 | .031 | .031 | .016 |
| 15781 | .008 | .031 | .031 | .016 |
| 15902 | .008 | .031 | .031 | .016 |
| 15991 | .008 | .031 | .031 | .016 |
| 16304 | .006 | .016 | .024 | .012 |
| 16547 | .008 | .031 | .031 | .016 |
| 15889 | .008 | .031 | .031 | .016 |
| 15928 | .008 | .031 | .031 | .016 |
| 16252 | .012 | .031 | .031 | .016 |
| 16395 | .008 | .016 | .016 | .008 |
| Avg. | .008 | .028 | .029 | .014 |

*Pen V = phenoxymethyl penicillin; Phen. = phenethicillin; Prop. = levo-propylcillin; Amino. = alpha-aminophenyl penicillin. Results are expressed as minimal inhibitory concentrations (μg/ml).

The activity of penicillin V and levopropylcillin is greater against pneumococci than that shown by either phenethicillin or methicillin (Table 3).

TABLE 3. *In vitro sensitivity of pneumococci to penicillins**

| Strain | Pen V | Phen. | Prop. | Meth. |
|---|---|---|---|---|
| ATCC 6301 | .003 | .025 | .003 | .006 |
| 6303 | .003 | .0125 | .006 | .003 |
| 6305 | .006 | .0125 | .003 | .0125 |
| 6308 | .003 | .003 | .003 | .003 |
| 8340 | .0125 | .0187 | .003 | .0187 |
| 10015 | .003 | .003 | .006 | .500 |
| 10359 | .003 | .025 | .003 | .500 |
| 10373 | .003 | .0125 | .003 | .500 |

*Pen V = phenoxymethyl penicillin; Phen. = phenethicillin; Prop. = levo-propylcillin; Meth. = methicillin. Results are expressed as minimal inhibitory concentrations ($\mu$g/ml).

The activity against nonpenicillinase-producing coagulase-positive staphylococci is compared in Table 4. Levopropylcillin and phenethicillin are equally effective; penicillin V is twice as active; but ten times as much methicillin is required to equal the least effective of the other three.

TABLE 4. *Antistaphylococcal effect of four penicillins* on nonpenicillinase-producing coagulase-positive staphylococci*

| Strain | Pen. V | Phen. | Prop. | Meth. |
|---|---|---|---|---|
| 209P | .003 | .025 | .05 | 0.78 |
| H27 | .003 | .003 | .05 | 1.56 |
| H2 | .003 | .0125 | .0125 | 0.78 |
| H21 | .009 | .006 | .025 | 1.56 |
| H185 | .0125 | .05 | .075 | 0.78 |
| H20 | .0125 | .0125 | .05 | 1.56 |
| H25 | .05 | .025 | .05 | 1.56 |
| H54 | .05 | .10 | .19 | 1.56 |
| H194 | .10 | .39 | .19 | 1.56 |
| H57 | .19 | .39 | .15 | 0.78 |
| Avg. | .04 | .10 | .08 | 1.25 |

*Pen V = phenoxymethyl penicillin; Phen. = phenethicillin; Prop. = levo-propylcillin; Meth. = methicillin. Results are expressed as minimal inhibitory concentrations ($\mu$g/ml).

Coagulase-positive staphylococci known to be resistant to penicillin V were studied for susceptibility to levopropylcillin and methicillin. Because of past reports of increasing resistance with large inocula, three types were used: light, with 125,000 organisms per ml of broth just prior to incubation; medium, or 1,250,000 per ml; and heavy, 12,500,000 per ml. With the light inocula, the action of penicillin V against the staphylococci was superior to that of phenethicillin, levopropylcillin, and methicillin. However, as the inoculum was increased, penicillin V became less effective, then phenethicillin, followed by levopropylcillin. Methicillin remained effective in approximately the same concentration with the heaviest inoculum as with the light (Tables 5 and 6).

TABLE 5. *Effect of three penicillins* on various inocula of penicillinase-producing staphylococci*

| Strain | Pen. V | Prop. | Phen. |
|---|---|---|---|
| $10^{-4}$ *Dilution* | | | |
| 209P | 0.003 | 0.04 | 0.04 |
| A149 | 1.56 | 0.4 | 0.4 |
| H456 | 6.25 | 1.56 | 1.17 |
| Myers | 6.25 | 0.78 | 1.17 |
| Kelly | 1.17 | 0.78 | 0.78 |
| $10^{-3}$ *Dilution* | | | |
| 209P | 0.003 | 0.02 | 0.01 |
| A149 | 100 | 6.25 | 50 |
| H456 | 100 | 9.37 | 50 |
| Myers | 100 | 12.5 | 100 |
| Kelly | 100 | 3.1 | 50 |
| $10^{-2}$ *Dilution* | | | |
| 209P | 0.003 | 0.04 | 0.01 |
| A149 | 100 | 100 | 100 |
| H456 | 100 | 100 | 100 |
| Myers | 100 | 100 | 100 |
| Kelly | 100 | 75 | 100 |

*Pen V = phenoxymethyl penicillin; Phen. = phenethicillin; Prop. = levo-propylcillin. Results are expresed as minimal inhibitory concentrations ($\mu$g/ml).

*Serum inactivation and enhancement.* Table 7 illustrates the concentrations of levopropylcillin and methicillin required to inhibit four strains of penicillin-resistant staphylococci and two strains of penicillin-sensitive staphylococci in both broth and 90% serum. With penicillin-resistant staphylococci, it appears that serum tended to increase the activity of levopropylcillin, whereas penicillin-sensitive bacteria were less susceptible in serum. The activity of methicillin against the sensitive and resistant staphylococci in broth and serum was very similar, suggesting that the effect of the methicillin on the organism is primarily through a single mechanism of action.

TABLE 6. *Effect of four penicillins* on various inocula of penicillinase-producing staphylococci*

| Strain | Pen. V | Phen. | Prop. | Meth. |
|---|---|---|---|---|
| *$10^{-4}$ Dilution* | | | | |
| H176 | 0.19 | 0.19 | 0.15 | 1.56 |
| H8 | 0.19 | 0.19 | 0.19 | 1.56 |
| K145 | 0.19 | 0.19 | 0.15 | 0.78 |
| H5 | 0.39 | 0.19 | 0.39 | 1.56 |
| H239 | 2.34 | 1.17 | 0.39 | 1.56 |
| H126 | 2.34 | 1.17 | 0.39 | 1.56 |
| H114 | 3.14 | 1.17 | 0.78 | 1.56 |
| *$10^{-3}$ Dilution* | | | | |
| H176 | 6.25 | 0.39 | 0.78 | 1.56 |
| H8 | 12.50 | 2.34 | 0.58 | 1.56 |
| H145 | 12.50 | 1.56 | 0.58 | 1.56 |
| H5 | 50.00 | 12.50 | 3.12 | 1.17 |
| H239 | 75.00 | 12.50 | 1.56 | 3.12 |
| H126 | 100.00 | 25.00 | 3.12 | 3.12 |
| H114 | 100.00 | 50.00 | 6.25 | 3.12 |

*Pen V = phenoxymethyl penicillin; Phen. = phenethicillin; Prop. = levo-propylcillin; Meth. = methicillin. Results are expressed as minimal inhibitory concentrations ($\mu$g/ml).

TABLE 7. *Comparison of antistaphylococcal activity* of levopropylcillin and methicillin in broth and serum*

| Strain | Inoculum | Levopropylcillin | | Methicillin | |
|---|---|---|---|---|---|
| | | Broth | 90% Serum | Broth | 90% Serum |
| *Resistant* | | | | | |
| H456 | $10^{-2}$ | 10 | 7.5 | 1.25 | 2.5 |
| | $10^{-3}$ | 10 | 3.75 | 1.25 | 2.5 |
| | $10^{-4}$ | 1.87 | 2.5 | 1.25 | 1.87 |
| A149 | $10^{-2}$ | 10 | 5 | 1.25 | 1.25 |
| | $10^{-3}$ | 10 | 5 | 1.87 | 0.93 |
| | $10^{-4}$ | 1.25 | 3.75 | 1.87 | 0.93 |
| Myers | $10^{-2}$ | 10 | 5 | 1.25 | 3.75 |
| | $10^{-3}$ | 10 | 5 | 1.87 | 1.25 |
| | $10^{-4}$ | 3.75 | 1.25 | 1.25 | 0.62 |
| Kelly | $10^{-2}$ | 10 | 2.5 | 1.87 | 0.93 |
| | $10^{-3}$ | 10 | 1.25 | 1.87 | 0.47 |
| | $10^{-4}$ | 1.87 | 0.62 | 1.87 | 0.24 |
| *Sensitive* | | | | | |
| 209P | $10^{-2}$ | 0.04 | 0.93 | 1.25 | 1.87 |
| | $10^{-3}$ | 0.04 | 0.62 | 1.25 | 0.93 |
| | $10^{-4}$ | 0.04 | 0.62 | 1.25 | 0.62 |
| C203 | $10^{-2}$ | 0.03 | 0.31 | 0.08 | 0.31 |
| | $10^{-3}$ | 0.02 | 0.31 | 0.16 | 0.31 |
| | $10^{-4}$ | 0.02 | 0.31 | 0.08 | 0.24 |

*Minimal inhibitory concentrations ($\mu$g/ml).

*Antibacterial activity and blood levels.* Figure 6 illustrates the high concentrations achieved in the serum with levopropylcillin. These are almost twice those of phenethicillin and four times those obtained with penicillin V. On the basis of estimates of absorption of penicillin V, from blood-level and urine-excretion studies (Fig. 5), levopropylcillin must be more than 80% absorbed within the first 6 to 8 hr.

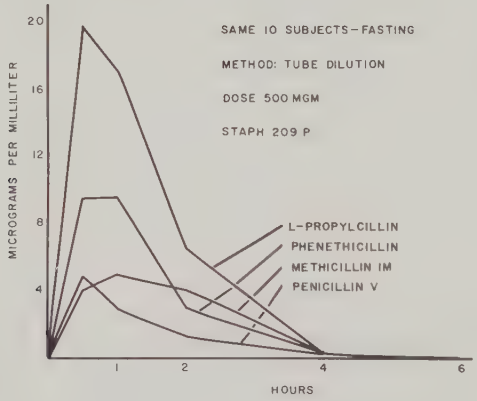


FIG. 6. *Comparison of penicillin serum concentrations.*

As has been discussed in previous papers, blood levels expressed in $\mu$g/ml are no indication of the relative effectiveness of an antibiotic unless the sensitivity of the pathogen is also known. The relative effectiveness can be demonstrated by selecting a representative pathogen and measuring the antibacterial activity in the serum of subjects who have taken each of the antibiotics. The results of such a determination are shown in Fig. 7. A coagulase-positive staphylococcus (209P) was found to have the same ratio of susceptibility to levopropylcillin, penicillin V, phenethicillin, and methicillin as seven other strains of nonpenicillinase-producing staphylococci. The peak value of antibiotic activity for each of these antibiotics was 330, 288, 277, and 4 $\mu$g, respectively. From this, we can conclude that, even though high levels of levopropylcillin are obtained in serum, its relative ineffectiveness against the staphylococci reduces its antibacterial action in the serum to approximately that of penicillin V and phenethicillin. However, there is not likely to be discernible difference among these three preparations in the treatment of infections due to penicillin-sensitive staphylococci.

From the data in Fig. 7, we must also conclude that methicillin probably should not be used in preference to penicillin V, phenethicillin,

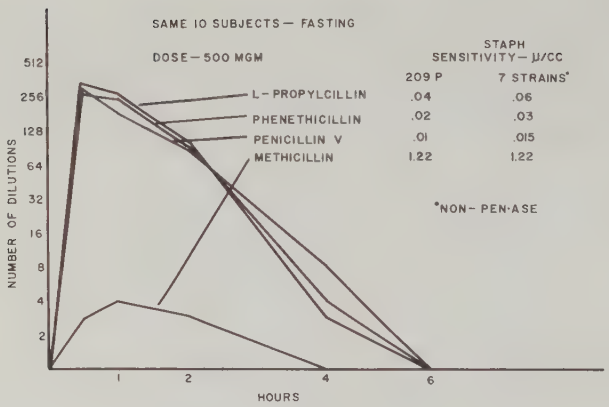


FIG. 7. *Antibacterial activity of sera from ten subjects in a crossover study.*

or levopropylcillin when the causative organism is a nonpenicillinase-producing penicillin-sensitive staphylococcus.

*Blood levels obtained with multiple doses.* The peak serum concentration during the second day of administration of 500 mg of levopropylcillin every 6 hr was twice as high as that obtained following the first dose (Fig. 8). The lowest concentration, prior to the next dose, with both the first and the fifth dose, was the same, indicating that there was no significant cumulation. The marked increase in peak concentration with the fifth dose suggests that, with repeated dosage, maximal "binding" by the serum has been exceeded, or the body "pool" has been saturated.

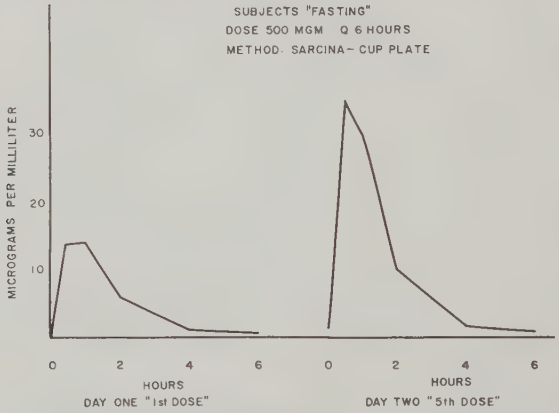


FIG. 8. *Effect on serum concentration of multiple doses of levopropylcillin.*

*Blood levels obtained with single doses.* A single dose of 500 mg of levopropylcillin gave serum concentrations that were twice those of 250 mg (Fig. 9). However, a 1,000-mg dose gave peak values that were four times those obtained after the 500-mg dose. This disproportionate increase in serum concentration, like the higher peak concentrations achieved with repeated dosage, might also be explained on the basis that the greater amounts administered finally exceeded maximal "protein binding" or serum "inactivation" capacity.

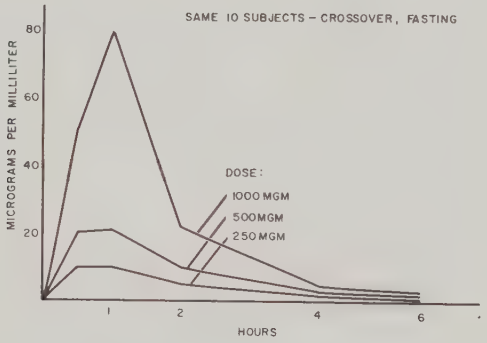


FIG. 9. *Effect of various single doses of levopropylcillin on serum concentration.*

*Blood levels of levopropylcillin when fasting and when taken with food.* As with penicillin V, levopropylcillin appears more gradually, has a lower peak concentration, and a longer duration of measurable levels when administered with food than when given to fasting patients (Fig. 10).

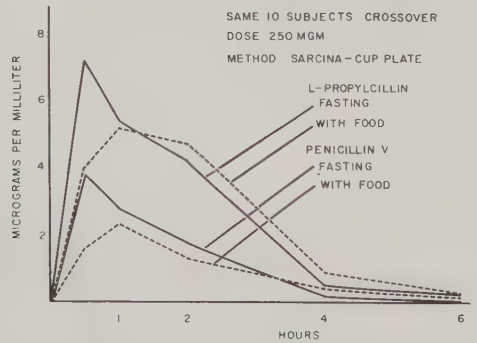


FIG. 10. *Effect of ingestion of food on serum levels of levopropylcillin.*

*Blood levels and serum antibacterial activity of penicillin V, levopropylcillin, and alpha-aminophenyl penicillin.* Levopropylcillin produced peak levels approximately four times as great as those obtained with penicillin V and alpha-aminophenyl penicillin. If the descending curve for alpha-aminophenyl penicillin is extrapolated, serum concentrations would persist for an additional 2 hr (Fig. 11). The peak serum antibacterial activity (Fig. 12) of penicillin V and levopropylcillin practically coincide, but are almost four times those of alpha-aminophenyl penicillin.

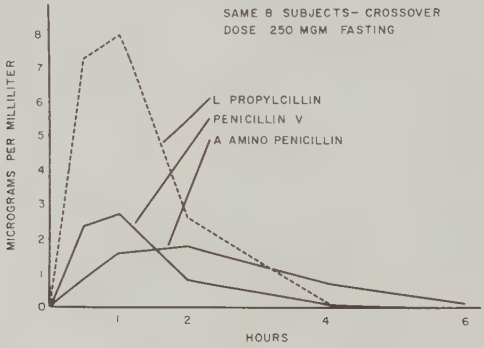


FIG. 11. *Persistence in serum of levopropylcillin, penicillin V, and alpha-aminophenyl penicillin.*

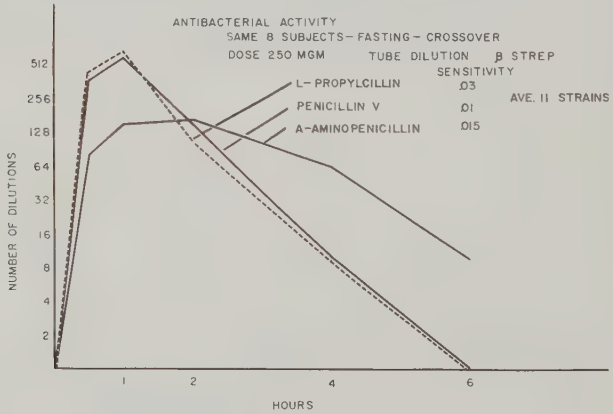


FIG. 12. *Peak serum concentrations of levopropylcillin, penicillin V, and alpha-aminophenyl penicillin.*

## Discussion

The levo diastereoisomer of alpha-phenoxypropyl penicillin, levopropylcillin, is better absorbed after oral administration than is the dextro-rotatory form. The absorption of levopropylcillin, as indicated by serum concentration and urine excretion, is greater than 80% in 6 to 8 hr, suggesting that it is entirely absorbed after oral administration.

This antibiotic is less active in vitro than are phenethicillin and penicillin V. This lower activity of levopropylcillin is offset by its greater absorption. It is as active as penicillin V in serum against beta-hemolytic streptococci, pneumococci, and sensitive staphylococci. Actual measurement of the activity of levopropylcillin and penicillin V in the serum of subjects, using nonpenicillinase-producing staphylococci and beta-hemolytic streptococci as indicator organisms, verifies that they are equally effective.

The high concentrations of levopropylcillin achieved with large single oral doses and with multiple dosage suggest possible therapeutic effectiveness in some instances of infections due to penicillinase-producing staphylococci. However, with the highest inocula studied in vitro, concentrations of levopropylcillin greater than those obtained with single 1-g oral doses were required for inhibition of the penicillin-resistant staphylococci. Present thinking would suggest that it is inappropriate to use penicillinase-susceptible penicillins in the therapy of infections due to penicillinase-producing staphylococci. This conclusion may be modified should subsequent studies verify our findings that serum enhances the action of penicillinase-susceptible penicillins against pencillin-resistant staphylococci.

A possible explanation for the paradox of serum enhancement of levopropylcillin activity against resistant staphylococci and serum inhibition of its action against penicillin-sensitive organisms may be made on the basis of two mechanisms of action of penicillin: prevention of maintenance of the cell wall (Cooper and Rowley, 1949; Cooper, 1955; Mathenson and Strominger, 1961) and inhibition of nucleic acid metabolism (Gale, 1952). In broth the penicillin-sensitive staphylococcus is easily killed with very low concentrations, by the inhibition of cell-wall synthesis. If penicillin-sensitive staphylococci are placed in serum, penicillin is not as effective against the cell-wall mechanism and must work against the internal nucleic acid metabolism. The latter requires higher penicillin concentrations (Gale, 1952).

Penicillin-resistant staphylococci probably are resistant to the action of penicillin against its cell wall (Elek, 1959). Therefore, the antistaphylococcal action must be directed toward the internal mechanism. Obviously, if the penicillin is ineffective against the cell wall of the resistant staphylococcus in broth, and the cell wall of sensitive staphylococci is not susceptible in serum, then practically the same concentration of the penicillin would be needed.

One may carry this theory further by suggesting that the action of methicillin is not against the cell wall; it may act by inhibition of internal nucleic acid metabolism. This would explain the similar concentrations required for both levopropylcillin and methicillin to be effective against penicillin-resistant staphylococci in broth and serum.

In solutions containing large quantities of penicillinase, the penicillin may be inactivated before it can inhibit the penicillin-resistant staphylococcus. This might explain the ineffectiveness of levopropylcillin in very high inocula of penicillin-resistant staphylococci.

The concentrations of levopropylcillin, when administered with food, are adequate to permit its use in therapy of infections without regard to meals.

This interesting penicillin has several properties that warrant careful clinical studies and comparison with other penicillins now available for investigation.

## Acknowledgments

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## Literature Cited

Cooper, P. D., and D. Rowley. 1949. Investigations with radioactive penicillin. Nature **163**:480-481.

Cooper, P. D. 1955. The site of action of penicillin: some properties of the penicillin-binding component of *Staphylococcus aureus*. J. Gen. Microbiol. **12**:100-107.

Elek, S. D. 1959. Staphylococcus pyogenes and its relation to disease, p. 443. E. & S. Livingstone Ltd., Edinburgh.

Gale, E. F. 1952. Points of interference of antibiotics in the assimilation of amino acids by bacteria, p. 5-20. *In* Mode d'action des antibiotiques. 2nd Intern. Congr. Biochem.

Kolmer, J. A. 1945. Penicillin therapy including streptomycin, tyrothricin and other antibiotic therapy, 2nd ed., p. 57. D. Appelton-Century Co., Inc., New York.

Mathenson, S., and J. Strominger. 1961. Effect of penicillin on the biosynthesis of the cell walls of *E. coli* and *Staphylococcus aureus*. J. Pharmacol. Exptl. Therap. **131**:1.

Rammeklamp, C. R. 1942. A method for determining the concentrations of penicillin in body fluids and exudates. Proc. Soc. Exptl. Biol. Med. **51**:95.

Williamson, G. M., J. K. Morrison, and K. J. Stevens. 1961. A new synthetic penicillin PA-24B. Lancet **1**:847.

# ALPHA-PHENOXYPROPYL PENICILLIN AND PROPIONYL ERYTHROMYCIN AGAINST RESISTANT STAPHYLOCOCCI

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Alpha-phenoxypropyl penicillin[1] (hereafter referred to as APP) is a new semisynthetic penicillin which appears to be active against strains of *Staphylococcus aureus* resistant to penicillin.

The present studies were carried out to assess the possible value of this antibiotic and to compare its activity with other available antibiotics and combinations of antibiotics against strains of *S. aureus*. Of 775 strains of coagulase-positive *S. aureus* isolated from patients, 100 strains that were highly resistant to penicillin and propionyl erythromycin (hereafter referred to as PE) were selected for the present experiments.

## Materials and Methods

Antibiotic sensitivity tests were carried out on blood agar streak plates containing serial twofold dilutions of the antibiotics. Trypticase soy agar (pH 7.3) was the base medium used, and to this was added 4% citrated human blood. All plates were made up the day of use. Before inoculation, plates were sectioned with a wax pencil so that six organisms could be streaked on each plate. The inoculum used consisted of one loopful (3 mm diam) from a 1:100 dilution of a 24-hr broth culture of heavy growth; the inoculum therefore consisted of approximately 500,000 organisms. Plates were then incubated for 20 to 24 hr at 37 C. The lowest antibiotic concentration showing no growth was recorded as the end point. Plates containing combinations of antibiotics were prepared in the same manner as described above, except that two antibiotics in equal quantities were incorporated in the media.

[1]Kindly supplied by Eli Lilly and Co.

## Results

During the past 2 years, we have tested 775 strains of coagulase-positive *S. aureus*. Of these, roughly 56% were sensitive to penicillin and 83% were sensitive to PE; 100 strains that were highly resistant to both penicillin and PE were used in the present study. The results of the sensitivity tests are shown in Table 1.

TABLE 1. *Antibiotic sensitivity of 100 strains of Staphylococcus aureus*

| Antibiotic | Amount required to inhibit growth (μg or units/ml) | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 0.39 or less | 0.78 | 1.56 | 3.125 | 6.25 | 12.5 | 25 | 50 | 100 | 200 or more | 1000 or more | Total |
| P* | | | | | | 8 | 16 | 25 | 13 | 38 | | 100 |
| PE | | | | | | | | | | | 100 | 100 |
| P plus PE | | 2 | 12 | 36 | 45 | 3 | | 2 | | | | 100 |
| APP | 1 | 13 | 37 | 18 | 11 | 16 | 3 | 1 | | | | 100 |
| APP plus PE | 8 | 42 | 46 | 3 | 1 | | | | | | | 100 |
| Methicillin | | 3 | 76 | 20 | 1 | | | | | | | 100 |

*P = penicillin; PE = propionyl erythromycin; APP = alpha-phenoxypropyl penicillin.

Eight strains required 12.5 units, 16 required 25 units, 25 required 50 units, 13 required 100 units, and 38 required 200 or more units of penicillin/ml to inhibit growth of *S. aureus*. All of the 100 strains required 1,000 or more μg/ml of PE for inhibition. We (1960) previously reported that resistance and susceptibility to PE both appear to follow an almost all-or-none pattern.

We also found that a combination of equal parts of penicillin and PE results in marked enhancement of action against organisms that are resistant to either antibiotic used alone. Table 1 reveals that 95 of the 100 strains were completely inhibited by amounts of the combination that can be easily attained in the serum of patients receiving these antibiotics.

The results of the studies shown in Table 1 indicate that APP is fairly active against most strains of staphylococci that are resistant to penicillin and PE; 80 of the 100 strains were completely inhibited by 6.25 μg/ml or less. Nevertheless, 20 strains required 12.5 μg/ml or more. When equal parts of APP and PE were combined, marked enhancement of action occurred. In fact, 99 of the 100 strains were inhibited by 3.125 μg of each per ml. The combination appears to be bactericidal for 96 strains using 1.56 μg of each per ml. One strain required 6.25 μg/ml of each to inhibit. Thus it appears that all of the 100 strains were completely inhibited by amounts of APP and PE that could be attained in the serum of patients receiving the combination. It is further evident (Table 1) that the combination of APP and PE is as effective as methicillin. Actually the number of strains inhibited by a combination of 1.56 μg/ml APP and PE or less is greater than for methicillin.

*Serum inhibition studies.* In view of the in vitro evidence that combinations of penicillin and PE were bactericidal for strains of *S. aureus* known to be highly resistant to each of the antibiotics, and in view of the evidence that combinations of APP and PE were equally if not more effective, studies were carried out to determine whether the serum of patients receiving these combinations would show this effect. The tube dilution method was used to determine the antibacterial activity of the serum of patients who received penicillin alone, PE alone, and the combination of penicillin and PE. Likewise, serum was obtained from patients who received only APP and PE and from patients who were receiving a combination of APP and PE simultaneously. The serum of these patients was obtained approximately 1 hr after the last dose of the antibiotic or the combination of antibiotics. The patients received 500 mg of the antibiotic or the combination of the antibiotics. The serum of these patients was inoculated with the resistant strains of *S. aureus* and read after 22 to 24 hr incubation at 37 C. The final volume in each tube prior to incubation was 1.0 ml. The first tube contained undiluted serum. Subsequent dilutions of 1:2, 1:4, and so forth were made by adding broth. An organism control and serum control were run with each test. The size of the inoculum was identical to that used for the plate method already described. The undiluted serum and the dilutions that were read as clear were subcultured on blood agar plates.

The results of the serum-inhibition studies are shown in Tables 2 and 3. The serum of at least two patients was included in each study. The serum was inoculated with two different but highly resistant strains of *S. aureus*. The undiluted serum of patients receiving penicillin alone or PE alone did not inhibit growth, whereas the undiluted serum of patients who were receiving penicillin plus PE was read as clear (Table 2). Likewise, the undiluted serum of patients receiving APP alone was usually read as slightly cloudy,

TABLE 2. *Serum inhibition of Staphylococcus aureus, tube dilution method*

| Antibiotic | Undiluted | 1:2 | 1:4 | 1:8 |
|---|---|---|---|---|
| P* | Slightly cloudy | Cloudy | Cloudy | Cloudy |
| PE | Cloudy | Cloudy | Cloudy | Cloudy |
| P plus PE | Clear | Slightly cloudy | Cloudy | Cloudy |
| APP | Slightly cloudy | Cloudy | Cloudy | Cloudy |
| APP plus PE | Clear | Slightly cloudy | Cloudy | Cloudy |

*P = penicillin; PE = propionyl erythromycin; APP = alpha-phenoxypropyl penicillin.

whereas the undiluted serum of patients receiving APP plus PE was clear. The serum of patients receiving a combination of penicillin plus PE and the serum of patients receiving APP plus PE was slightly cloudy in the tubes which had been diluted 1:2.

TABLE 3. *Serum inhibition of Staphylococcus aureus, subcultures on blood agar plates**

| Antibiotic | Undil. | 1:2 | 1:4 | 1:8 | Control |
|---|---|---|---|---|---|
| P† | 2 + | 4 + | 4 + | 4 + | 4 + |
| PE | 4 + | 4 + | 4 + | 4 + | 4 + |
| P plus PE | 0 | 2 + | 3 + | 4 + | 4 + |
| APP | 1 + | 4 + | 4 + | 4 + | 4 + |
| APP plus PE | 0 to 1 | 3 + | 4 + | 4 + | 4 + |

*0 = no growth; 1 + = 1 to 10 colonies; 2 + = 10 to 100 colonies; 3 + = scant growth; 4 + = heavy growth.

†P = penicillin; PE = propionyl erythromycin; APP = alpha-phenoxypropyl penicillin.

The results of the subcultures on blood agar plates made from the tube dilution tests are shown in Table 3. A bactericidal effect was noted only in the undiluted serum of those patients receiving the combinations. Occasionally, a few colonies appeared on subcultures of the tubes of undiluted serum that were read as clear. A bacteriostatic effect was usually observed in the serum diluted 1:2 of patients receiving the combinations.

The in vitro studies herein reported suggest that highly resistant strains of *S. aureus* can be inhibited by the APP-PE combination. These studies further suggest that this combination of antibiotics is equal to if not more active than methicillin. Since APP and PE can be effectively administered by the oral route, whereas the use of methicillin is restricted to parenteral use, it seemed desirable to determine whether the combination of APP and PE could be effectively used in the treatment of infections due to highly resistant *S. aureus*. In the past, we have used the combination of penicillin and PE successfully in the treatment of resistant staphylococcal infections; however, this combination could not be used by patients known to be allergic to penicillin.

## Clinical Studies

We have had occasion to use APP in the treatment of only 16 patients. Since this clinical experience is limited, it is obvious that no definitive statement can be made concerning the ultimate role of APP in the treatment of staphylococcal infections.

In 10 of the 16 patients treated, APP was used alone. In five, APP was combined with PE. The average daily dose of APP used

in the first 10 patients was 1 g administered in amounts of 250 mg every 6 hr by the oral route. The duration of treatment varied from 5 to 10 days in eight of the patients. Two patients discontinued the antibiotic on the second day because of nausea and gastrointestinal irritation. Two other patients complained of nausea and abdominal discomfort after taking the medication but continued their course of treatment. One patient developed a maculopapular rash while taking the antibiotic, but this subsided immediately when the antibiotic was discontinued. This patient had never received penicillin or any other antibiotic before. The organisms isolated in this group of patients were sensitive to the antibiotic, and the results obtained were considered good or excellent in all eight of the patients that completed the treatment. This group of patients was treated with the first batch of APP available, and it was thought that the high incidence of gastrointestinal irritation was due to substances introduced during the process of extraction and was not due to APP. Subsequently, material extracted by a different method would on occasion produce slight nausea, but in no instance was it a troublesome reaction.

Six patients were treated with a combination of equal parts of APP and PE. The patients received 250 mg of each antibiotic every 6 hr. The average daily dose, therefore, was 2 g. The duration of treatment varied from 7 to 28 days. In two of these patients, the staphylococci isolated from large carbuncles were sensitive to both penicillin and PE. Both patients were treated for 7 days; clinical results were excellent, as would be expected. The staphylococcus isolated from a large axillary abscess in the third patient was highly resistant to penicillin and sensitive to PE. This organism was completely inhibited by APP in amounts of 6.25 $\mu$g/ml. The patient received APP alone for 4 days. The clinical response was not considered dramatic, although there was some improvement. Prompt healing and complete recovery occurred when the patient received a combination of APP plus PE for the subsequent 6 days of treatment.

We have had occasion to use the combination of APP plus PE in the treatment of three patients suffering with severe staphylococcal infections due to organisms resistant to most of the previously available antibiotics. All three patients had failed to respond to intensive treatment with either penicillin or PE. The data on these three patients are summarized in Table 4. The results would seem to justify a more detailed description of these patients.

*Case 14.* The patient was a 50-year-old female whose son was a physician. The son, his wife, and children had suffered with recurrent staphylococcal infections. The patient had cared for one of these children with recurrent boils. When seen, she was suffering with a large furuncle and cellulitis of the left thigh. She had failed to respond to previous antibiotic therapy. The *S. aureus* isolated required 200 units of penicillin for inhibition, and was resistant to 1,000 $\mu$g/ml of PE. The organism was inhibited by 6.25 $\mu$g/ml of

APP, and was completely inhibited by 1.56 μg/ml each of a combination of APP and PE. The patient received 500 mg of the combination every 6 hr for a total of 10 days. Complete recovery occurred and there has been no recurrence of the infection.

TABLE 4. *Treatment of resistant staphylococcal infections with APP plus PE**

| Case no. | Organism resistant to | Average daily dose | Days treated | Result |
|---|---|---|---|---|
| | | g | | |
| 14 | P PE | 2 | 10 | Complete recovery |
| 15 | P PE Vancomycin Novobiocin | 2 | 28 | Recovery; 2 sinuses cleared 1st week. All cleared 4th week. |
| 16 | P PE | 2 | 7 | Recovery |

*P = penicillin; PE = propionyl erythromycin; APP = alpha-phenoxypropyl penicillin.

*Case 15.* The patient was a 19-year-old male. After repair of a right inguinal hernia in his local community hospital, he developed a severe staphylococcal wound infection with necrosis of the right scrotum and cellulitis extending up over the entire abdomen and back to the thoracic cavity. He had received penicillin before admission to our service. Shortly after admission, he developed an extensive drug rash and urticaria due to penicillin. Extensive surgical drainage and debridement was carried out. Multiple sinuses were present and draining purulent material. The patient also developed furunculosis on the head, body, and legs. The *S. aureus* isolated required more than 200 units of penicillin/ml to inhibit. It required more than 1,000 μg/ml of PE to inhibit. The organism was also resistant to both novobiocin and vancomycin. It required 12.5 μg/ml of APP to inhibit. It was found that the organism was completely inhibited by 1.56 μg/ml each of the combination of APP plus PE. The patient received 250 mg of APP combined with 250 mg of PE every 6 hr for a total of 28 days. Treatment was carried out on an ambulatory basis and he attended college during the entire time. The response was excellent. Two of the three draining sinuses closed after the second week. Material from the third sinus was obtained for culture after 2 weeks of therapy, and the organism again was isolated but was still sensitive to the combination of APP and PE. After the 4th week, the third sinus closed and treatment was discontinued.

*Case 16.* The patient was a 32-year-old male. He was suffering with a severe staphylococcal pharyngitis. The throat was acutely inflamed and contained heavy exudate. Before admission, he had received 7 days treatment with penicillin administered by the oral

route. There had been no improvement. Cultures revealed *S. aureus*, which was resistant to both penicillin and erythromycin. He received 500 mg of the combination of APP plus PE for 7 days. Within 48 hr, there was marked improvement, followed by complete recovery.

## Literature Cited

Herrell, W. E., A. Balows, and J. Becker. 1960. Erythrocillin: a new approach to the problem of antibiotic-resistant staphylococci. Antibiotic Med. & Clin. Therapy 7:637-642.

# ANTISTAPHYLOCOCCAL ACTIVITY OF ALPHA-PHENOXYPROPYL PENICILLIN

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Alpha-phenoxypropyl penicillin has been reported to give higher blood levels of penicillin after oral administration (Williamson, Morrison, and Stevens, 1961) and to be more active in vitro against penicillinase-producing staphylococci than either penicillin V or phenethicillin (Jackson and Rao, 1961).

To further investigate this new penicillin, we have compared the in vitro activity of alpha-phenoxypropyl penicillin with three other penicillins against different inocula of both penicillinase-producing staphylococci and staphylococci which did not produce penicillinase. In vivo activity was tested by treating nasal carriers of staphylococci and correlating changes in quantitative nasal cultures with the penicillinase activity of staphylococci isolated from the nose and with antistaphylococcal activity of individual patients sera against different inocula of the strains carried.

## Materials and Methods

A total of 50 coagulase-positive staphylococci were isolated from lesions of patients at the Louisville General Hospital from January through May 1961.

The bacteriostatic and bactericidal activities of four penicillins were determined by tube-dilution methods (Petersdorf et al., 1960) in heart infusion broth using a final concentration of $10^{-2}$ and $10^{-4}$ dilutions of overnight cultures of staphylococci. The production of penicillinase was determined by a modified Gots method (Haight and Finland, 1952).

Hospitalized patients were cultured five times per week for staphylococci, by quantitative nasal cultures (White et al., 1959). Patients were classified as carriers when at least two of three pretreatment cultures contained coagulase-positive staphylococci. Carriers were treated with 250 mg of alpha-phenoxypropyl penicillin orally every 6 hr for 7 to 10 days.

After at least 24 hr of treatment, serum was obtained 1 hr after a dose of alpha-phenoxypropyl penicillin and assayed for antistaphylococcal activity by tube-dilution methods (White and Varga, 1961) against a fresh subculture of the strain isolated from the patient's nose before therapy. Significant inhibitory activity was considered present if visible growth was inhibited by 50% serum or less.

## Results

When light inocula of penicillinase-producing staphylococci were tested (Table 1), all strains were inhibited by 6.25 μg/ml of methicillin, by 12.5 μg/ml of alpha-phenoxypropyl penicillin, by 50 μg/ml of phenethicillin, and by 100 μg/ml of penicillin G. At a concentration of 6.25 μg/ml all strains were susceptible to methicillin, to alpha-phenoxypropyl penicillin (97%), to phenethicillin (77%), and to penicillin G (47%).

At a concentration of 6.25 μg/ml, methicillin was bactericidal for 87% of light inocula of penicillinase-producing staphylococci, alpha-phenoxypropyl penicillin for 47%, phenethicillin for 33%, and penicillin G for 30% of strains tested (Table 2).

TABLE 1. *Bacteriostatic activity of four penicillins against 50 recently isolated staphylococci*

| Strain type | Penicillins | Cumulative percentage susceptible of $10^{-4}$ dilution of staphylococci in μg/ml | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | 0.05 | 0.1 | 0.2 | 0.4 | 0.8 | 1.6 | 3.1 | 6.2 | 12.5 | 25 | 50 | 100 |
| Penicillinase producers (30 strains) | Alpha-phenoxypropyl | 3 | 10 | 17 | 33 | 47 | 83 | 93 | 97 | 100 | | | |
| | G | | | 10 | 23 | 40 | 43 | 43 | 47 | 57 | 80 | 97 | 100 |
| | Phenethicillin | 0 | 10 | 17 | 33 | 43 | 50 | 63 | 77 | 77 | 90 | 100 | |
| | Methicillin | | | | 0 | 3 | 10 | 73 | 100 | | | | |
| Nonpenicillinase producers (20 strains) | Alpha-phenoxypropyl | 15 | 20 | 100 | | | | | | | | | |
| | G | 55 | 100 | | | | | | | | | | |
| | Phenethicillin | 16 | 100 | | | | | | | | | | |
| | Methicillin | | | 0 | 5 | 30 | 95 | 100 | | | | | |
| All strains | Alpha-phenoxypropyl | 8 | 14 | 50 | 60 | 68 | 90 | 96 | 98 | 100 | | | |
| | G | 22 | 40 | 46 | 54 | 64 | 66 | 66 | 68 | 74 | 88 | 98 | 100 |
| | Phenethicillin | 8 | 46 | 50 | 60 | 66 | 70 | 78 | 86 | 86 | 94 | 100 | |
| | Methicillin | | | | 0 | 4 | 18 | 82 | 100 | | | | |

TABLE 2. *Bactericidal activity of four penicillins*

| Strain type | Penicillins | Cumulative percentage susceptible of $10^{-4}$ dilution of staphylococci in μg/ml | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | 0.05 | 0.1 | 0.2 | 0.4 | 0.8 | 1.6 | 3.12 | 6.25 | 12.5 | 25 | 50 | 100 |
| Penicillinase producers (30 strains) | Alpha-phenoxypropyl | | 0 | 3 | 10 | 17 | 23 | 33 | 47 | 63 | 73 | 80 | 97 |
| | G | | | 0 | 7 | 13 | 13 | 27 | 30 | 37 | 43 | 53 | 63 |
| | Phenethicillin | | | 0 | 7 | 10 | 17 | 23 | 33 | 37 | 50 | 50 | 57 |
| | Methicillin | | | | | 0 | 7 | 33 | 87 | 97 | 97 | 100 | |
| Nonpenicillinase producers (20 strains) | Alpha-phenoxypropyl | 5 | 20 | 60 | 90 | 90 | 100 | | | | | | |
| | G | 25 | 85 | 95 | 100 | | | | | | | | |
| | Phenethicillin | 15 | 55 | 90 | 100 | | | | | | | | |
| | Methicillin | | 0 | 0 | 5 | 5 | 15 | 40 | 90 | 95 | 95 | 100 | |
| All strains | Alpha-phenoxypropyl | 2 | 8 | 26 | 42 | 46 | 54 | 60 | 68 | 78 | 86 | 88 | 98 |
| | G | 10 | 34 | 38 | 44 | 48 | 48 | 56 | 58 | 62 | 66 | 72 | 78 |
| | Phenethicillin | 6 | 22 | 36 | 44 | 46 | 50 | 54 | 60 | 62 | 70 | 70 | 74 |
| | Methicillin | | | 0 | 2 | 2 | 10 | 36 | 88 | 96 | 96 | 100 | |

All staphylococci which did not produce penicillinase were inhibited by 0.1 μg/ml of penicillin G or phenethicillin, by 0.2 μg/ml of alpha-phenoxypropyl penicillin, and by 3.1 μg/ml of methicillin.

When heavier inocula of penicillinase-producing staphylococci were tested (Table 3), all strains were again inhibited by 6.25 μg/ml of methicillin, but only 37% were inhibited by alpha-phenoxypropyl penicillin, 7% by phenethicillin, and none by penicillin G at the same concentration.

TABLE 3. *Bacteriostatic activity of four penicillins against 50 recently isolated staphylococci*

| Strain type | Penicillins | Cumulative percentage susceptible of $10^{-2}$ dilution of staphylococci in μg/ml | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | 0.05 | 0.1 | 0.2 | 0.4 | 0.8 | 1.6 | 3.1 | 6.2 | 12.5 | 25 | 50 | 100 |
| Penicillinase producers (30 strains) | Alpha-phenoxypropyl | | | | | 0 | 10 | 20 | 37 | 40 | 43 | 43 | 43 |
| | G | | | | | | | | 0 | 3 | 3 | 10 | 20 |
| | Phenethicillin | | | | | | | 0 | 7 | 10 | 23 | 27 | 30 |
| | Methicillin | | | | | | 0 | 63 | 100 | | | | |
| Nonpenicillinase producers (20 strains) | Alpha-phenoxypropyl | 0 | 25 | 90 | 100 | | | | | | | | |
| | G | 60 | 90 | 100 | | | | | | | | | |
| | Phenethicillin | 35 | 90 | 100 | | | | | | | | | |
| | Methicillin | | | | 0 | 5 | 30 | 95 | 100 | | | | |
| All strains | Alpha-phenoxypropyl | 0 | 10 | 36 | 40 | 40 | 46 | 52 | 62 | 64 | 66 | 66 | 66 |
| | G | 24 | 36 | 40 | 40 | 40 | 40 | 40 | 40 | 42 | 42 | 46 | 52 |
| | Phenethicillin | 14 | 36 | 40 | 40 | 40 | 40 | 40 | 44 | 46 | 54 | 56 | 58 |
| | Methicillin | | | | 0 | 2 | 12 | 76 | 100 | | | | |

Heavy inocula of staphylococci which did not produce penicillinase were inhibited at approximately the same concentrations as light inocula.

*Effect of alpha-phenoxypropyl penicillin on nasal carriers.* Patients who were nasal carriers of coagulase-positive staphylococci were treated with 250 mg of alpha-phenoxypropyl penicillin every 6 hr.

Of the 25 patients treated, 13 were carriers of penicillinase-producing strains and 12 of strains resistant to 50 μg/ml of penicillin G, when larger inocula were tested. Although light inocula of all 13 strains were sensitive to 1.6 μg/ml or less of alpha-phenoxypropyl penicillin, 5 of the 13 strains were resistant to 100 μg/ml when larger inocula were used.

The remaining 12 patients were carriers of staphylococci which did not produce penicillinase and which were susceptible to 3.12 μg/ml or less of either penicillin G or alpha-phenoxypropyl penicillin, when large inocula were tested.

The mean carrier rate in patients whose strains did not produce penicillinase fell from 100% before treatment to 12% on the seventh day of treatment (Table 4). No significant change occurred in the carrier rate of patients from whom penicillinase-producing staphylococci were isolated before treatment.

In treated patients whose sera inhibited both light and heavy inocula of their own nasal staphylococci, carrier rates fell from 100%, before therapy, to 20% by the ninth day of treatment (Table 5).

TABLE 4. *Effect of alpha-phenoxypropyl penicillin on nasal carriers of staphylococci which produced penicillinase as compared to carriers of staphylococci which did not produce penicillinase*

| | Nonpenicillinase producers | | Penicillinase producers | |
|---|---|---|---|---|
| Treatment day | No. cultured | Positive | No. cultured | Positive |
| | | % | | % |
| -3 | 3 | 100 | 4 | 100 |
| -2 | 10 | 100 | 12 | 100 |
| -1 | 12 | 84 | 12 | 100 |
| 1 | 8 | 50 | 11 | 91 |
| 2 | 8 | 50 | 8 | 88 |
| 3 | 1 | 0 | 5 | 80 |
| 4 | 5 | 20 | 5 | 80 |
| 5 | 8 | 25 | 7 | 100 |
| 6 | 8 | 38 | 10 | 80 |
| 7 | 8 | 12 | 10 | 80 |

No consistent alterations of carrier rates were observed in patients whose sera inhibited light inocula, but did not inhibit heavy inocula of staphylococci isolated from the nose before therapy.

TABLE 5. *Effect of alpha-phenoxypropyl penicillin on carriers whose sera inhibited heavy inocula of staphylococci as compared to carriers whose sera inhibited light inocula only*

| | $10^{-4}$ and $10^{-2}$ dilutions | | $10^{-4}$ dilution | |
|---|---|---|---|---|
| Treatment day | No. cultured | Positive | No. cultured | Positive |
| | | % | | % |
| -3 | 3 | 100 | 1 | 100 |
| -2 | 11 | 100 | 5 | 100 |
| -1 | 11 | 82 | 4 | 100 |
| 1 | 9 | 89 | 3 | 100 |
| 2 | 7 | 72 | 3 | 67 |
| 3 | 3 | 100 | 3 | 67 |
| 4 | 4 | 50 | 2 | 50 |
| 5 | 8 | 50 | 2 | 100 |
| 6 | 10 | 50 | 4 | 75 |
| 7 | 8 | 38 | 5 | 80 |
| 8 | 6 | 33 | 4 | 100 |
| 9 | 5 | 20 | 2 | 100 |

## Discussion

With the availability of 6-aminopenicillanic acid in large quantities, a number of different side chains have been attached, to produce penicillins with different degrees of resistance to penicillinase. Of these, methicillin is effective in vitro against both light and heavy inocula of penicillinase-producing staphylococci (White and Varga, 1961).

Phenethicillin is more effective against both light and heavy inocula of penicillinase-producing staphylococci than penicillin G, but the concentration required to inhibit heavy inocula is higher than the blood levels obtained after usual therapy.

As shown in this study, alpha-phenoxypropyl penicillin is more active than phenethicillin against heavy inocula of penicillinase-producing staphylococci. However, the inhibitory concentration is markedly increased with heavy inocula as compared to light inocula.

Although all staphylococci from nasal carriers were inhibited by 1.6 μg/ml of alpha-phenoxypropyl penicillin when light inocula were tested, treatment with alpha-phenoxypropyl penicillin was highly effective in eradicating staphylococci only from cultures of staphylococci which did not produce penicillinase.

Antistaphylococcal activity against nasal staphylococci could be demonstrated in the sera of 17 carriers during treatment with alpha-phenoxypropyl penicillin. Only in the 11 patients whose sera also inhibited the growth of heavy inocula of their own nasal staphylococci could a significant decrease in mean carrier rates be demonstrated.

These data suggest that in vivo activities of penicillins are more closely correlated with in vitro activity of sera against heavy inocula of staphylococci rather than light inocula.

## Literature Cited

Haight, T. H., and M. Finland. 1952. Modified Gots test for penicillinase production. Am. J. Clin. Pathol. **22**:806-808.

Jackson, F. L., and K. K. Rao. 1961. Comparative in vitro activities of five penicillins against penicillinase-producing staphylococci. Lancet **1**:850-851.

Petersdorf, R. G., M. C. Rose, H. R. Minchew, W. R. Kene, and I. L. Bennett, Jr. 1960. The sensitivity of hemolytic staphylococci to a series of antibiotics. II. A three-year progress report. A. M. A. Arch. Internal Med. **105**:398-412.

White, A., T. Hemmerly, M. P. Martin, and V. Knight. 1959. Studies on the origin of drug-resistant staphylococci in a mental hospital. Am. J. Med. **25**:26-39.

White, A., and D. T. Varga. 1961. Antistaphylococcal activity of dimethoxyphenyl penicillin, p. 146-159. *In* P. A. Bunn, [ed.], A symposium on dimethoxyphenyl penicillin. Syracuse University Press, Syracuse.

Williamson, G. M., J. K. Morrison, and K. J. Steven. 1961. A new synthetic penicillin PA-248. Lancet **1**:847-850.

# SOME MISCELLANY ABOUT d-ALPHA-AMINOBENZYL PENICILLIN

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This study summarizes experiences with d-alpha-aminobenzyl penicillin (P-50) in the laboratory and in the management of a few patients with gram-negative bacterial infections (P-50 is marketed as Penbritin by Beecham Laboratories in England. It was supplied by Bristol Laboratories, Inc., Syracuse, N.Y.). This work follows recently published studies by a group of British investigators who observed that the drug has a high degree of usefulness in certain clinical infections (Rolinson and Stevens, 1961; Knudsen, Rolinson, and Stevens, 1961; Brown and Acred, 1961; Stewart et al., 1961). As this new penicillin has the unique property of inhibiting, in vitro at least, the growth of a number of gram-negative microorganisms, further observations seem necessary to describe more precisely its efficacy.

## Experimental

Penicillin P-50 (Fig. 1) is an acid-stable, partially synthesized substance which is active against a number of gram-positive and gram-negative microorganisms. Because, like penicillin G, it is hydrolyzed to the inactive penicilloic acid by penicillinase, because, on a weight basis, it is somewhat less active than penicillin G, and because it is far more difficult to produce than penicillin G, its major antibacterial advantage rests with its ability to inhibit other than gram-positive bacteria and cocci. It shows a high degree of activity

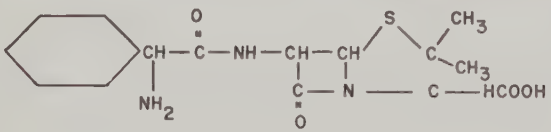


FIG. 1. *Structure of penicillin P-50.*

against all species of *Salmonella, Haemophilus influenze, Escherichia coli, Klebsiella,* and many species of *Proteus.* It is not active against most strains of *Aerobacter aerogenes* and *Pseudomonas pyocyanea.* In vitro observations show that the new agent is considerably more potent, on a weight basis, than the tetracyclines or chloramphenicol. Susceptibilities of the sensitive gram-negative bacilli range from 0.6 to 5.0 μg per ml. Many gram-negative organisms, which like *Aerobacter* produce penicillinase in appreciable amounts, are completely resistant to the drug. Nonetheless, the agent has a broader range of activity than penicillin G, but like G it is highly bactericidal in its action.

Measurement of concentrations of P-50 is performed by the usual cup-plate method, using *Sarcina lutea* as the test organism. After a single dose of 750 mg, peak concentrations in serum are attained within 2 hr; levels above 1.0 μg/ml are maintained for somewhat less than 6 hr (Table 1). Similar levels are observed after the intramuscular administration of 500 mg. Peak concentrations in serum after six hourly doses are as high as 12 to 15 μg/ml and at no time are they less than 0.8 μg/ml. Urinary excretion within 8 hr after administration is 25% (orally) and 40% (parenterally).

TABLE 1. *Blood serum levels obtained with penicillin P-50*

| Method of administration | Peak level | | Level after 6 hr | Level after 6 hourly doses |
|---|---|---|---|---|
| | Time | Amount | | |
| | *min* | *μg/ml* | *μg/ml* | *μg/ml* |
| Intramuscular* | 20-30 | 8.0 | 0.8 | 0.8-15 |
| Oral+ | 60 | 8.5 | 1.0 | 2.0-12 |

*Dose: 500 mg.
+Dose: 750 mg.

Theoretically, these levels are sufficient to provide adequate amounts of P-50 to inhibit growth of susceptible bacteria in vivo. The discrepancy between the dose of drug given and the less than 50% recoverable in urine has not been explained. Although large amounts are excreted in bile (Brown and Acred, 1961), this alone does not account for the loss of 50% of a given dose.

*Use of P-50 in human infections caused by gram-negative bacteria.* Eighteen adults, ranging in age from 20 to 87, with acute infections severe enough to require hospitalization, were treated solely with P-50. Of the 18 patients, 17 had genitourinary-tract infections; the other had sepsis from an unrecognized focus, presumably in the gall bladder. Underlying diseases in 17 were chronic or incurable. The present infection was not necessarily the first which the patient had had; indeed, the majority gave long histories of recurring infections with multitudes of past treatments, including the use of a number of antimicrobial agents. Each infection in this series was

bacteriologically proven, and each was associated with hyperpyrexia and other major symptoms. Three patients had minimal to moderate elevations of blood urea nitrogen; one other had seriously impaired renal function. The degree of morbidity was hard to assess. Only two of the acute infections were life-threatening; the others were disabling but not fearful. Obviously, any acute process superimposed upon the chronic underlying disease became important to every patient, so that favorable clinical responses were imperative.

Table 2 lists the causative microorganisms and kinds of disease treated. In 15 of the 18, a single species of bacteria was present in the urine; a mixture was found twice. The infecting organism was found solely in the blood in one. Tests of susceptibility to P-50 revealed that 15 of the isolated strains were inhibited by the drug in concentrations of less than 5.0 μg/ml. In the other three (two cases with *P. pyocyanea* and one with *Aerobacter*), resistance was measured in the range between 50 and 100 μg/ml. Other than the initial isolates, no further observations were recorded upon bacteria subsequently recovered.

TABLE 2. *Fifteen patients with acute infections treated with d-alpha-aminobenzyl penicillin*

| Organism | No. patients | Underlying disease |
|---|---|---|
| *Salmonella* | 2 | Chronic pyelonephritis (2) |
| *Escherichia coli* | 8* | Acute prostatitis<br>Chronic pyelonephritis (4)<br>Acute prostate, with obstruction (2)<br>Actue general infection, primary focus unrecognized<br>Relaxed pelvic floor with marked cystitis and large residual |
| *Proteus* | 3 | Urethral stricture<br>Benign prostatic hypertrophy with obstruction (2) |
| *Aerobacter aerogenes* | 1 | Ca. bladder with obstruction |
| *Pseudomonas pyocyanea* | 2 | Benign prostatic hypertrophy urethral stricture |
| Mixture of *E. coli*, *Proteus*, and *Aerobacter* | 2 | Both with chronic pyelonephritis with urethral calculi and obstruction |

*Two with positive blood cultures.

The dosage of drug used routinely was 750 mg to 1 g, four times a day. The oral route was used exclusively in 13 of the 18. In five, the same total daily dose of drug was started parenterally; after 3 or 4 days, a shift to the oral route was prescribed. Treatment extended from 4 to 24 days, the extent of therapy being dependent in the main upon the seriousness of the underlying process. Relapses occurred both clinically and bacteriologically in six patients, 7 to 30 days after cessation of therapy.

Clinical results achieved were eminently satisfactory in 16 of the 18 patients. In one, the outcome could not be evaluated fairly. In the other, who was the most critically ill of the entire group, treatment was stopped too soon to determine efficacy (see below).

Bacteriological responses were measurable in all 18 patients and in each it was both impressive and rapid, including the changes observed in the two patients whose clinical responses were not particularly impressive. The six relapses occurred in patients with impossibly damaged kidneys and urethras. Although microorganisms in the urine were cleared transiently with therapy, late recurrences were anticipated and inevitably developed. No relapse occurred while the patients were under treatment. A second course of treatment with P-50 in three of the six patients was followed by the same performance, i.e., bacteriological clearing and symptomatic cures.

No serious untoward side effects from the drug itself were observed. Two patients with histories of prior allergy to penicillin G exhibited no difficulties with the new agent, despite its continued use for 9 and 17 days, respectively. Some of the patients noted mild flatulence and diarrhea; this did not interfere with continued treatment.

Table 3 depicts the bacteriological changes in two patients, in one of whom the infecting bacteria presumably were resistant to the drug in vitro. In general, cultures of urine from the other patients followed the same pattern: marked reduction in colony numbers between the 1st and 2nd days, and virtual sterility by the 4th day. There were three exceptions to this; in each instance there was severe bladder obstruction from carcinoma of the prostate or urethral strictures. In these, organisms persisted in urine for as long as 7 days before bacterial counts below 100 colonies/ml were observed. Speed of clearing of the urine in infections caused by *P. pyocyanea*, also resistant in vitro, was no different.

TABLE 3. *Changes in degree of bacilluria as influenced by administration of amino benzyl penicillin to patients with acute urinary tract infections*

| | Patient 1 | Patient 2 |
|---|---|---|
| Day 1 | No. colonies *A. aerogenes* per ml urine* | No. colonies *E. coli* per ml urine |
| *hr* | | |
| 0 | 1:100,000 dil T.N.C.* | 1,900,000 |
| 0-6 | T.N.C. | T.N.C. |
| 6-12 | 90,000 | – |
| 12-24 | 150,000 | 250,000 |
| Day 2 | 18,000 | 88 |
| Day 4 | 440 | 1 |
| Day 7 | 6 | – |

*Organism resistant to 50 μg aminobenzyl penicillin/ml. TNC: too numerous to count.

Brief summaries of four patients are presented to demonstrate the range of responses. They include the two impossible of evaluation, and two others with unusually acute infections which responded well.

H. H. was a 50-year-old man with chronic pyelonephritis and mild uremia. In addition, he had had a carcinoma of the prostate, which had been removed only partially 3 years previously; there was an appreciable bladder-neck obstruction. He had developed multiple acute urinary-tract infections in the 2 years before the present episode. In every acute attack of infection, *Salmonella* had been the only organism isolated from his urine. Each attack in the past had been temporarily controlled with either tetracycline or chloramphenicol. On the present occasion, he entered the hospital with a temperature of 104 F, which had been preceded by a shaking chill. He was acutely ill, with no evidence of shock. His urinary-tract symptoms of dysuria, frequency, and burning had become worse during the previous 4 days. He was given P-50, 750 mg orally, four times daily. Fever and symptoms were controlled promptly, but the urine continued to show *Salmonella* for 72 hr and pyuria persisted. After 96 hr, the urine cleared, and therapy was stopped on the 7th day. He remained well for the next 7 days, after which there was a prompt and severe relapse. The same organism was recovered from the urine, in counts exceeding 100,000 colonies/ml. A second course of therapy again was satisfactory; *Salmonella* disappeared from the urine in 72 hr. However, the pyuria persisted for the next 17 days and dysuria was not appreciably lessened; treatment was finally discontinued at this time. He left the hospital 10 days later, and there has been no follow-up. It is likely that, despite the exquisite in vitro susceptibility of this strain of *Salmonella*, the drug did not alter in any degree his basic infectious problem. It did alter impressively and favorably his intercurrent clinical disease.

The second patient, C. R., aged 55, had chronic and frequently recurring acute urinary-tract infections which resulted from a congenital stricture of the urethra. For years, this required dilatations at least weekly. For the previous 8 years, the three or four yearly severe infections had been associated with either a species of *Proteus* or *P. pyocyaneus*. In the current instance, he developed abrupt onset of fever, unusually prominent pyuria, and major signs and symptoms of an acute infection. For the following 9 days he was treated with presumably adequate amounts of chloramphenicol and a sulfonamide. These failed to initiate clinical or bacteriological response (*Proteus*). P-50 1 g four times a day, was followed by a prompt remission of both; the response was truly impressive. The drug was stopped after 5 days. Four days later there was an acute relapse, albeit considerably less severe, and, on this occasion, *P. pyocyaneus* was isolated. A second 4-day course of P-50 provoked another

impressive disappearance of symptoms, and the urine was cleared of all organisms. He was well for the next 60 days.

A third patient, J. C., aged 40, entered the hospital with a febrile illness and abnormal urine. He was desperately ill and in peripheral vascular shock. Because localization of the infection was not secure and because cultures were lost initially, he was given large doses of penicillin G and streptomycin for 48 hr. There was no response. Subsequently, a diagnosis of *E. coli* sepsis was made and P-50 was started, intramuscularly, 1 g four times a day. The response was most gratifying; the drug was stopped on the fifth day. In an effort to explain more fully the patient's underlying disease, he was catheterized 5 days later. The procedure proved to be quite traumatic, for an enlarged soft and obstructing prostate was discovered. Within 8 hr there was recurrence of high fever (105 F), with shock and bloody urine contaminated with *E. coli*. P-50 was given orally, 1 g four times a day, and again there was a prompt remission. Drug was continued for 7 days, during which the prostatitis subsided. There was no subsequent relapse.

In both these last two cases, initial therapy was probably stopped too soon. Had it been continued for 7 to 12 days, the relapses might have been averted.

The last patient, a 24-year-old woman, may represent a drug failure. She entered the hospital desperately ill with sickle cell trait, acute hemolysis, and a temperature of 104.5 F. No diagnosis of infection was made for 3 days. No specific therapy was started until the fourth hospital day, when the initial cultures were reported positive for *E. coli*. In this period her clinical picture worsened and she was morbidly ill. No localization of the primary infection was found. Her urine was not remarkably abnormal, although first cultures did show *E. coli*. In retrospect, it can be presumed that she had acute cholecystitis and cholangitis with no stones. P-50, 1 g four times daily, intramuscularly, was administered for 72 hr (Fig. 2). Initially, her temperature came down slightly but she remained critically ill; the clinical picture was essentially unchanged. Because we feared death, kanamycin was started, 1.5 g, three times daily. Febrile crisis occurred in 36 hr, and thereafter clinical improvement was rapid. She made an uneventful recovery.

In retrospect (easy to be sure), blood and urine were cleared of *E. coli* before kanamycin was started. Blood samples taken periodically from this patient during the administration of the penicillin revealed concentrations ranging between 2.6 to almost 10 μg/ml. The infecting organism was sensitive to less than 2.5 μg/ml.

## Discussion

It would be quite convenient to have a penicillin that is active in vivo against infections caused by gram-negative microorganisms.

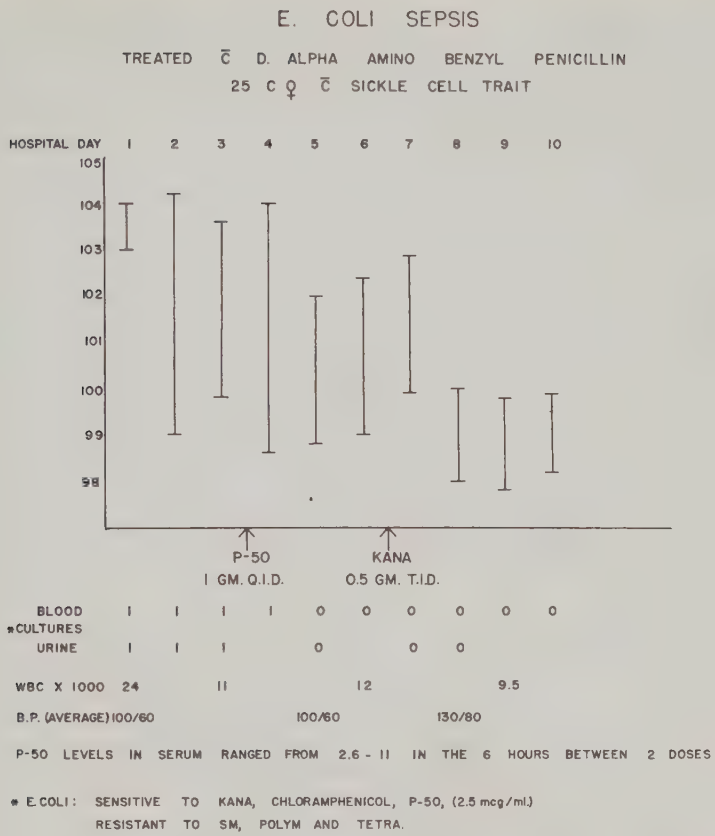
E. COLI SEPSIS
TREATED C̄ D. ALPHA AMINO BENZYL PENICILLIN
25 C ♀ C̄ SICKLE CELL TRAIT
HOSPITAL DAY 1 2 3 4 5 6 7 8 9 10
105
104
103
102
101
100
99
98
P-50
1 GM. Q.I.D.
KANA
0.5 GM. T.I.D.
BLOOD *CULTURES 1 1 1 1 0 0 0 0 0 0
URINE 1 1 1 0 0 0
WBC X 1000 24 11 12 9.5
B.P. (AVERAGE) 100/60 100/60 130/80
P-50 LEVELS IN SERUM RANGED FROM 2.6 - 11 IN THE 6 HOURS BETWEEN 2 DOSES
* E.COLI: SENSITIVE TO KANA, CHLORAMPHENICOL, P-50, (2.5 mcg/ml.)
RESISTANT TO SM, POLYM AND TETRA.

FIG. 2. *Temperature response to treatment with penicillin P-50 and kanamycin of a patient with Escherichia coli septicemia.*

Perhaps P-50 supplies part of that convenience, but its place as the drug of choice in severe infections caused by susceptible gram-negative bacteria cannot yet be recommended. Our single case of apparent failure may be indicative, because adequate blood concentrations had been achieved and maintained above the in vitro level of sensitivity of the infecting organism, for the vast majority of hours each day. She apparently had an acute superficial infection; at least the resolution was complete after therapy with kanamycin was instituted, and no residual infectious lesions were diagnosed. This case actually spoils otherwise respectable results in a series of acute infections, all of whom responded satisfactorily, particularly

bacteriologically. There was only one other individual in the series whose infection was nearly as severe, and he relapsed after what was considered an adequate first course of the drug. Consequently, the only recommendation possible at this time is to await further clinical trials, perhaps with higher doses for morbidly ill individuals.

Higher doses of P-50 than we were accustomed to giving in this series of patients may be necessary in certain cases. An appreciable amount (in excess of 35%) of the drug is bound to serum protein. In this state it presumably is not available for antibacterial activity in vivo. Similarly, P-50 is rapidly excreted in urine, so that it remains for a relatively short time in effective concentrations in serum. If the infection being treated is associated with extra large numbers of organisms, or, as was true in most of our cases, there is a compromised blood supply to the area of inflammation, effective amounts of drug may not be attained or maintained. It is conceivable that this series of events could explain the prompt relapses in some of our cases and perhaps also the discouraging result in the one case that did not respond well in the first 72 hr of treatment. In the latter instance, because of hemolysis and other deteriorated body-defense mechanisms, together with overwhelming numbers of *E. coli*, (uninhibited for 96 hr), the amount of drug finally administered (4 g daily) was not sufficient to control the infectious part of her disease. Had we prescribed 6 g per day or even more and had we continued that amount for another 24 hr, a more satisfactory response might have been anticipated. In any event, before a final evaluation of its clinical efficacy is made, further studies of time-dose relationships in gram-negative bacillary infections are required. And there is an urgent need to learn more about its tissue distribution in the human. A considerable amount of each dose of the drug is apparently lost somewhere or is metabolized; this lost material may be crucial to its eventual usefulness in vivo.

Regimens of treatment with P-50 cannot now be described safely. It is suggested that for acute infections at least 4 g daily by mouth is a minimum. More is surely required for seriously ill patients with a chronic underlying primary disease. Somewhat less may be needed if given parenterally. The total daily amount must be divided into at least four, and preferably six, single doses. In general, this schedule provides approximately 50 mg per kg of body weight per day for adults. On the other hand, from experiences recorded by pediatricians (Stewart et al., 1961; Ross, *personal communication*), a daily dose of 100 mg/kg is required for children; daily doses of 150 to 200 mg/kg have been prescribed. These have been given without untoward side effects. There seems to be no logical reason that these larger amounts could not be given to adults also.

Three of our patients had urinary-tract infections caused by bacteria resistant in vitro to between 50 and 100 $\mu$g/ml. Satisfactory clinical and bacteriological results obtained in all three deserves

comment, for theoretically there should have been none. Exceedingly high concentrations are found in urine, in excess of 500 μg/ml for several hours after each dose. These concentrations could inhibit or kill presumably resistant organisms in the urine; in our cases, this advantage apparently was sufficient to provoke a clinical remission. It must be emphasized, contrariwise, that mere reduction of numbers of bacteria in urine probably does not have an overall favorable influence upon basic kidney infections. Bacteriological "cure" in urine is not necessarily the *sine qua non* of cure of disease. This phenomenon also deserves further study.

This new penicillin offers great hope for the development of an even better one. There can be no question that P-50 is exceedingly active in vitro, actually surpassing the potency of chloramphenicol and tetracycline to inhibit the growth of and to kill certain gram-negative bacteria. One can speculate that inevitably a new penicillin can be so chemically organized that it will be active not only against the spectrum of organisms now killed by penicillin G, but also that it will not be inactivated by penicillinase. With these properties it might be the only antibacterial agent needed in the clinical practice of medicine. The successive developments of cephalosporin N, dimethoxyphenyl penicillin, methylphenylisoxazolyl penicillin, and finally, penicillin P-50, permit the presumption that this ideal may yet be found.

### Acknowledgment

This work was supported by Bristol Laboratories.

### Literature Cited

Brown, D. M., and P. Acred. 1961. "Penbritin"—a new broad-spectrum antibiotic: preliminary pharmacology and chemotherapy. Brit. Med. J. 2:197-198.

Knudsen, E. T., G. N. Rolinson, and S. Stevens. 1961. Absorption and excretion of "Penbritin." Brit. Med. J. 2:198-200.

Rolinson, G. N., and S. Stevens. 1961. Microbiological studies on a new broad-spectrum penicillin, "Penbritin." Brit. Med. J. 2:191-196.

Stewart, G. T., H. M. T. Coles, H. H. Nixon, and R. J. Holt. 1961. "Penbritin": an oral penicillin with broad-spectrum activity. Brit. Med. J. 2:200-206.

# STUDIES ON α-AMINO BENZYL PENICILLIN

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α-Amino benzyl penicillin (P-50) is a new synthetic penicillin prepared by conjugation of *d*-α-amino phenyl acetic acid with 6-aminopenicillanic acid (Rolinson and Stevens, 1961). It is of special interest because of its claimed efficacy against gram-negative organisms (Rolinson and Stevens, 1961; Stewart et al., 1961). Furthermore, its stability to acid permits oral as well as parenteral use.

The present report deals with the in vitro antimicrobial activity of P-50 against a variety of gram-positive and gram-negative bacteria and its efficacy in the treatment of 24 patients with infections due to gram-negative bacilli. Sixteen of these patients had failed to respond to prior antibiotic therapy. The remaining eight patients were treated with P-50 from the beginning. On the basis of in vitro data, an attempt was made to select patients with infections due to strains of *Escherichia coli* and *Proteus* sp. This study is still in progress.

## Materials and Methods

*In vitro studies.* A total of 100 strains of gram-positive and gram-negative bacteria, including fresh clinical isolates as well as stock strains, was tested for sensitivity in vitro by a twofold serial tube dilution method (Schweinburg, Sylvester, and Rutenburg, 1954). These strains were distributed as follows: *E. coli*, 28; *Proteus* sp., 21; enterococci, 8; *Salmonella paratyphi*, 5; *Pseudomonas aeruginosa*, 3; *Shigella dysenteriae*, 2; *Alcaligenes faecalis*, 2; *Klebsiella pneumoniae*, 2; *Bacillus subtilis*, 2; and *Sarcina lutea*, 3. The concentrations of P-50 (supplied by Bristol Laboratories, Syracuse, N.Y.) ranged from 0.18 to 25 μg/ml. Each tube was inoculated with a $10^{-3}$ dilution of an 18- to 24-hr broth culture of the particular strain to be tested. The tubes were examined after 24 hr and again after 48 hr of incubation at 37 C. In reading the tubes, the last clear tube

after 24 hr usually remained clear on reincubation for an additional 24 hr. In a few instances, there was a shift of one tube to the next higher concentration of the drug. The subcultures of 0.1 ml of the last clear tube showed no growth in most instances. A few subcultures, however, yielded several colonies and these tubes became slightly cloudy on reincubation. This shift, however, was never more than one tube. Therefore, the absence of any significant difference between the bacteriostatic or bactericidal concentrations suggests that in vitro the antibacterial action of P-50 was bactericidal.

The results (Table 1) indicate that most strains of *E. coli*, *Proteus* sp., *Salmonella*, *Shigella*, and *S. faecalis* were sensitive to P-50, i.e., inhibited by 6.2 μg/ml or less. *A. aerogenes*, *P. aeruginosa*, and *K. pneumoniae* were uniformly resistant. Of staphylococci, strains which produced penicillinase were resistant and those which did not were sensitive. In comparison to penicillin G, P-50 was considerably less effective against penicillin G-sensitive microorganisms.

TABLE 1. *Sensitivity of various strains within a bacterial species to α-amino benzyl penicillin**

| Species | No. strains tested | Concentrations of P-50 | | |
|---|---|---|---|---|
| | | 0.1 to 1.56 μg/ml | 3.12 to 6.25 μg/ml | 12.5 to 25 μg/ml |
| *E. coli* | 28 | 13 | 11 | 4 |
| *Proteus* sp. | 21 | 19 | 1 | 1 |
| *S. paratyphi* | 8 | 5 | 0 | 3 |
| *A. aerogenes* | 8 | 1 | 0 | 7 |
| *S. faecalis* | 8 | 6 | 1 | 1 |
| *S. aureus* | 8 | 1 | 0 | 7 |
| *S. typhosa* | 5 | 5 | 0 | 0 |
| *Pseudomonas* | 3 | 0 | 0 | 3 |
| *S. dysenteriae* | 2 | 2 | 0 | 0 |
| *K. pneumoniae* | 2 | 0 | 0 | 2 |
| *A. faecalis* | 2 | 1 | 0 | 1 |
| *S. lutea* | 3 | 1 | 2 | 0 |
| *B. subtilis* | 2 | 2 | 0 | 0 |

*In last clear tube after 24 hr.

α-Amino benzyl penicillin was effective in vitro against most strains of *E. coli*, *Proteus* sp., *A. faecalis*, *Salmonella*, and *S. faecalis*. The drug was not effective against the other gram-negative bacteria tested. P-50 was less active than penicillin G against staphylococci, streptococci (Rolinson and Stevens, 1961; Stewart et al., 1961), and other gram-positive bacteria sensitive to penicillin G.

*Clinical observations*. Patients with a variety of infections (Table 2) received P-50. Of the 24 studied, 16 had failed to respond

to previous antibiotic therapy, single or in combination (streptomycin in five patients, tetracycline in eight, and chloramphenicol in four). Gram-negative bacteria alone were isolated in 17 patients, and in combination with staphylococci in five, and in two patients no cultures were obtained. The bacterial strains isolated were tested for sensitivity in vitro. Most strains of *E. coli* and *Proteus* sp. were found to be sensitive, and staphylococci (penicillinase producers),

TABLE 2. *Data on 23 patients with surgical infections treated with α-amino benzyl penicillin*

| Diagnosis | Wound culture | Previous treatment | Duration of treatment | Results after treatment | |
|---|---|---|---|---|---|
| | | | | Bacteriological | Clinical |
| | | | *days* | | |
| Septicemia, urinary infection* | *E. coli* | – | 8 | Sterile | Indeterminate |
| Septicemia, urinary infection | *E. coli* | – | 9 | Sterile | Good |
| Urinary infection | *E. coli*<br>*P. vulgaris* | – | 7 | Sterile | Good |
| Urinary infection | *P. vulgaris*<br>*S. aureus* | – | 27 | *S. aureus* | Good |
| Urinary infection | *E. coli* | Penicillin, streptomycin, Declomycin | 8 | – | Good |
| Urinary infection | *E. coli* | – | 27 | Sterile | Good |
| Urinary infection | *E. coli* | Methicillin, Kanamycin | 3 | *P. aeruginosa* | Good |
| Urinary infection | *E. coli*<br>*A. aerogenes* | – | 25 | *A. aerogenes* | Good |
| Urinary infection | *E. coli* | Gantrisin | 7 | *P. aeruginosa* | Good |
| Urinary infection | *A. aerogenes* | Streptomycin, tetracycline | 7 | *A. aerogenes* | Poor |
| Urinary infection | *A. aerogenes* | Streptomycin, chloramphenicol | 8 | *A. aerogenes* | Poor |
| Urinary infection | *A. aerogenes* | Streptomycin, chloramphenicol | 10 | *A. aerogenes* | Poor |
| Septic shock, pancreatitis | *P. morganii*<br>*S. aureus* | Tetracycline | 2 | *S. aureus* | Poor |
| Septic shock, pancreatitis | *A. aerogenes*<br>*E. coli* | Tetracycline | 10 | *E. coli*<br>*A. aerogenes* | Poor |
| Septic shock, septicemia, and pneumonia* | *E. coli*<br>*A. aerogenes* | – | 11 | *A. aerogenes* | Poor |
| Septic shock, septicemia after cystoscopy | *E. coli*<br>*P. morganii* | Chloramphenicol | 6 | Sterile | Indeterminate |
| Wound infection | *P. vulgaris*<br>*S. aureus* | Tetracycline | 7 | *S. aureus* | Good |
| Wound infection | *P. vulgaris*<br>*S. aureus* | Prostaphlin | 4 | *S. aureus* | Good |
| Wound infection | *P. vulgaris* | – | 10 | *S. aureus* | Good |
| Wound infection, subhepatic abscess | *E. coli* | Tetracycline | 12 | Sterile | Good |
| Wound infection perianastamotic abscess | *E. coli* | Tetracycline | 7 | – | Good |
| Acute cholecystitis | – | Tetracycline | 9 | – | Good |
| Diverticulitis* | – | Tetracycline | 7 | – | Indeterminate |
| Pyopneumothorax† | *E. coli*<br>*P. vulgaris*<br>*S. aureus* | Streptomycin, penicillin, chloramphenicol | 34 | *P. vulgaris* | Good |

*Indeterminate - concomitant other antibiotics.

†Combined therapy with 5-methyl-3-phenyl-4-isoxazolyl penicillin.

*A. aerogenes*, and *Pseudomonas* were resistant. Twelve patients had urinary tract infections, associated with positive blood cultures in two, and four developed septic shock due to the severity of their infection (acute pancreatitis in two, septicemia and pneumonia in one, and septicemia following cystoscopy in the fourth patient). Three patients had postoperative wound infections, and two had postoperative wound infections and intraperitoneal abscesses. The remaining three patients had pyopneumothorax, acute cholecystitis, and diverticulitis, respectively (Table 2).

Following oral administration, P-50 was rapidly absorbed. After oral administration of a 1-g dose, serum specimens were collected and assayed for P-50 activity. (These assays were performed by J. Lannon and Kay Casson, Control Division, Bristol Laboratories, Syracuse, N.Y.) These results and those of other investigators (Rolinson and Stevens, 1961; Stewart et al., 1961) indicated average serum concentrations of 3.2 μg/ml at 1 hr, 5.7 μg/ml at 2 hr, and 1.8 μg/ml at 4 hr. At the end of 6 hr, virtually no detectable antibiotic was present in the serum. In patients given 1 g of P-50 orally every hr, serum concentrations were 3.8 to 14 μg/ml at 1 hr, 1.1 to 4.9 μg/ml at 2 hr, 0.9 to 4.1 μg/ml at 3 hr, 1.7 to 3.0 μg/ml at 4 hr, and 0.3 to 1.0 μg/ml at 6 hr. The drug was excreted in high concentrations in the urine in 1 to 3 hr. Urine concentrations ranged as high as 1,100 μg/ml. After intramuscular administration higher concentrations were achieved in the serum for shorter periods because of more rapid excretion.

Of the 24 patients, 18 received 1 g of P-50 orally four times daily for 3 to 27 days. Six acutely ill patients who could not take oral medication received 250 to 500 mg intramuscularly (5) or intravenously (1) every 6 hr during the acute phase of their disease, and then were shifted to the oral dosage form. The usual duration of therapy was 7 to 12 days. One patient developed severe glossitis and epigastric distress, and another an allergic rash typical of a penicillin reaction. There were no evidences of renal, hepatic, or hematopoietic toxicity.

## Results

The results of therapy could not be evaluated in three patients because of concomitant administration of other antibiotics, and were deemed indeterminate. Six others failed to respond. The remaining 15 patients showed a satisfactory clinical and bacteriological result (Table 3).

Strains of *E. coli* and *Proteus* sp. were usually eliminated in the course of therapy (Table 4). *P. aeruginosa*, *A. aerogenes*, and penicillin G-resistant staphylococci persisted when present, or

TABLE 3. *Clinical results of P-50 therapy*

| Diagnosis | No. patients | Results | | |
|---|---|---|---|---|
| | | Good | Poor | Indeterminate |
| Urinary tract infections<br>Septicemia (2)* | 12 | 8 | 3 | 1 |
| Septic shock<br>Pancreatitis (2)<br>Septicemia (2) | 4 | 0 | 3 | 1 |
| Soft tissue infections<br>Postoperative wound infections (3)<br>Intraperitoneal abscesses (2) | 5 | 5 | 0 | 0 |
| Cholecystitis | 1 | 1 | 0 | 0 |
| Pyopneumothorax | 1 | 1 | 0 | 0 |
| Diverticulitis | 1 | 0 | 0 | 1 |
| Totals | 24 | 15 | 6 | 3 |

*Numbers in parentheses refer to no. of patients with more than one infection diagnosed.

TABLE 4. *Bacteriological results of P-50 therapy in 22 patients*

| Microorganism | No. eliminated | No. not eliminated | Superinfection |
|---|---|---|---|
| *E. coli* | 13* | 1 | 0 |
| *Proteus* sp. | 7† | 1 | 0 |
| *A. aerogenes* | 0 | 6 | 0 |
| *S. aureus* | 0 | 5 | 1 |
| *P. aeruginosa* | 0 | 0 | 2 |

*Two patients received other antibiotics concomitantly.
†One patient received other antibiotics concomitantly.

appeared in the course of P-50 administration and required supplementary antibiotic therapy. Thus, nine patients with persistant *Aerobacter aerogenes* or staphylococci were given kanamycin or 5-methyl-3-phenyl-4-isoxazolyl penicillin (Bristol Laboratories, Syracuse, N.Y.), a new synthetic penicillin effective orally against resistant staphylococci (Rutenburg et al., *in press*).

Twelve of the 24 patients had urinary tract infections (Table 2) due to *E. coli* alone (6) or in combination with *Proteus vulgaris* (1), or *A. aerogenes* (1), *P. vulgaris* and *Staphylococcus aureus* (1), and *A. aerogenes* (3). In two patients the same microorganism (*E. coli*) was recovered from the blood stream. The three patients with *A. aerogenes* infection showed no clinical or bacteriological response. A fourth patient was cured but the result deemed indeterminate because of concomitant use of another antibiotic. The remaining eight patients showed subsidence of the signs and symptoms of their infection. In four of these, the urine was sterilized; *A. aerogenes* and staphylococci persisted in two, although *E. coli* or *P. vulgaris* were

eliminated; two developed asymptomatic superinfections due to *P. aeruginosa*. Both patients with *E. coli* septicemia showed defervescence, sterilization of the blood, and subsidence of signs and symptoms in 48 to 72 hr.

Results of P-50 therapy in the four patients with septic shock were difficult to evaluate. One had pancreatitis, a pancreatic fistula, and wound infection. He showed striking clinical improvement without any change in the bacteriological findings, and had progressive healing with eventual closure of the fistula and healing of the wound, unrelated to antibiotic therapy. The second patient failed to respond because of undrained loculated intraperitoneal sepsis. The third patient with septicemia and pneumonia died of pulmonary embolism. The role of P-50 therapy could not be assessed in the fourth patient with septicemia because of concomitant therapy with chloramphenicol.

Five patients had soft-tissue infections; three infections were in postoperative wounds; two of them showed clearance of the inciting microorganism (*E. coli* or *Proteus* sp.), decreased drainage, and prompt healing of the wound. The third patient developed a superinfection with resistant staphylococci which were cleared with 5-methyl-3-phenyl-4-isoxazolyl penicillin (Rutenburg et al., *in press*). The remaining two patients had infected wounds and intraperitoneal sepsis due to *E. coli* (a subhepatic abscess and a perianastomotic abscess). Both responded with decreased drainage, defervescence, and wound healing.

The patient with acute cholecystitis was so critically ill that he was not deemed suitable for surgery. With P-50 he showed dramatic defervescence and subsidence of his signs and symptoms (Fig. 1).

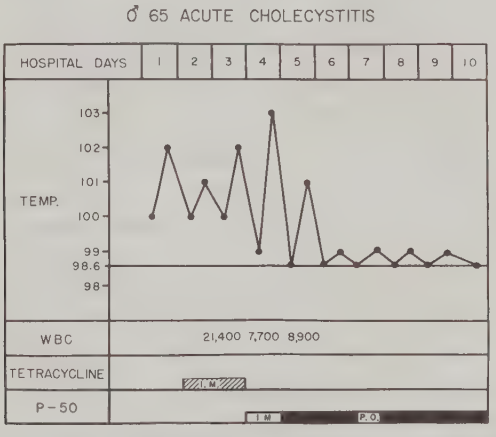


FIG. 1. *P-50 therapy in 65-year-old man with acute cholecystitis.*

He recovered without operative intervention. The patient with diverticulitis recovered with P-50 and kanamycin therapy. The last patient was an acutely ill, debilitated 68-year-old man with pyopneumothorax due to *P. vulgaris* and *S. aureus* (Fig. 2). His recovery was slow, but progressive. *P. vulgaris* was suppressed with P-50, but he also required 5-methyl-3-phenyl-4-isoxazolyl penicillin (Prostaphlin) for control of the staphylococcal infection.

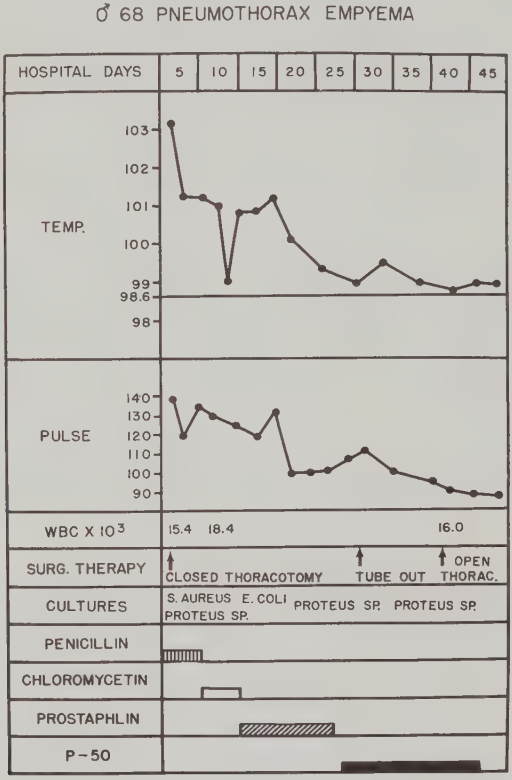


FIG. 2. *P-50 therapy in 68-year-old man with acute pyopneumothorax.*

## Discussion

The derivation of the basic structure of penicillin (Sheehan, Henery-Logan, and Johnson, 1953; Sheehan and Ferris, 1959; Sheehan

and Henery-Logan, 1959) and the subsequent biosynthetic isolation of 6-aminopenicillanic acid by Batchelor et al. (1959) led to the preparation of a variety of semisynthetic penicillins in the laboratory (Rolinson and Stevens, 1961; Rolinson et al., 1960; Perron et al., 1960; Abstracts of Conference, 1961). Two of these, 2,6-dimethoxyphenyl penicillin and 5-methyl-3-phenyl-4-isoxazolyl penicillin have been found to be resistant to penicillinase and thus effective both in vitro and clinically against resistant staphylococci (Rutenburg et al., *in press;* Rutenburg, Greenberg, and Schweinburg, 1960). α-Amino benzyl penicillin is another modified penicillin which is of particular interest because of its efficacy against some gram-negative bacteria. Thus, many strains of *E. coli*, *Proteus* sp., and *Salmonella* were inhibited by low concentrations of the drug, whereas *A. aerogenes* and *P. aeruginosa* were not. The resistance of the latter microorganisms as well as that of *S. aureus* stems from instability to penicillinase which destroys this penicillin (Rolinson and Stevens, 1961; Stewart et al., 1961).

The antimicrobial spectrum of the drug seemed limited in vitro largely to strains of *E. coli*, *Proteus* sp., *Salmonella*, and *S. faecalis*. The clinical data herein reported indicate that α-amino benzyl penicillin was effective in the control of some but not all infections due to *E. coli* and *Proteus* sp. since the use of this drug in several patients critically ill with surgical infections due to coliform organisms alone or along with staphylococci was not associated with clinical and bacteriological cure.

Infections of the urinary tract due to either *E. coli* or *Proteus* sp., including two with associated septicemia, responded well. All five patients with postoperative wound infections showed eradication of the initial responsible organisms and healing of the wound. The prompt recovery of the patient with acute cholecystitis may be related to the high levels of the drug achieved in the liver and bile (Stewart et al., 1961).

In all cases, appropriate surgical management, with removal of foreign bodies, adequate drainage, and debridement was carried out as indicated.

P-50 was easily absorbed from the gastrointestinal tract producing serum bactericidal levels in 1 hr, which were sustained for about 3 to 4 hr. The drug was well tolerated. In critically ill patients, or those unable to tolerate oral medication, it could be given parenterally. After parental administration, the therapeutically effective serum concentrations were achieved within 0.5 to 1 hr.

Most infections due to gram-positive bacteria can be controlled with penicillin G; resistant staphylococcal infections are controlled with either of two semisynthetic penicillins (methicillin and 5-methyl-3-phenyl-4-isoxazolyl penicillin). Now a third penicillin, effective against several species of gram-negative bacteria, has become available. This may well be the first step leading to the effective control

of infections due to gram-negative bacteria with semisynthetic penicillins. Thus, we may postulate the eventual synthesis of new penicillin derivatives specific against many other microorganisms.

## Literature Cited

Batchelor, F. R., R. P. Doyle, H. H. C. Nayler, and G. N. Rolinson. 1959. Synthesis of penicillin: 6-aminopenicillanic acid in penicillin fermentations. Nature **183**:257-258.

Conference on 5-methyl-3-phenyl-4-isoxazolyl penicillin (Prostaphlin). 1961. Bristol Laboratories, Syracuse.

Perron, Y. G., L. B. Crast, W. J. Gottstein, W. F. Minor, and L. C. Cheney. 1960. Synthesis of penicillin-152 [potassium (α-phenoxyethyl) -penicillin] and both diastereoisomers. Antibiotics Ann. 1959-1960, p. 107-110.

Rolinson, G. N., and S. Stevens. 1961. Microbiological studies on a new broad-spectrum penicillin, "Penbritin". Brit. Med. J. **2**:5246-5251.

Rolinson, G. N., S. Stevens, F. R. Batchelor, J. C. Wood, and E. B. Chain. 1960. Bacteriological studies on a new penicillin – BRL-1241. Lancet **2**:564-567.

Rutenburg, A. M., H. L. Greenberg, and F. B. Schweinburg. 1960. Clinical experiences with 2,6-dimethoxyphenyl penicillin monohydrate in staphylococcal infections. New Engl. J. Med. **263**:1174-1178.

Schweinburg, F. B., E. M. Sylvester, and A. M. Rutenburg. 1954. Relation of in vitro sensitivity test to clinical response to Aureomycin therapy. Surgery **35**:204-212.

Sheehan, J. C., and J. P. Ferris. 1959. The chemical conversion of penicillin G into a biologically active synthetic penicillin series. J. Am. Chem. Soc. **81**:2912.

Sheehan, J. C., and K. R. Henery-Logan. 1959. A general synthesis of the penicillins. J. Am. Chem. Soc. **81**:5838-5839.

Sheehan, J. C., K. R. Henery-Logan, and D. A. Johnson. 1953. The synthesis of substituted penicillins and simpler structural analogs. VII. The cyclization of a penicilloate derivative to methyl phthalimidopenicillanate. J. Am. Chem. Soc. **75**:3292-3293.

Stewart, G. T., H. M. T. Coles, H. H. Nixon, and R. J. Holt. 1961. "Penbritin": an oral penicillin with broad-spectrum activity. Brit. Med. J. **2**:5255-5261.

# COMPARATIVE STUDIES OF FIVE NEW ORAL PENICILLINS

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In the present study, three new oral penicillins, phenoxypropyl, phenoxyisopropyl, and phenoxyisobutyl penicillin, have been compared in vitro and in vivo with phenoxymethyl (penicillin V) and phenoxyethyl penicillin (phenethicillin). Other new oral penicillins, P-12 and SKF 12141, with chemical structures somewhat similar to methicillin, have also been studied and compared from the standpoint of in vitro activity and absorption after oral administration.

## Materials and Methods

Phenoxypropyl and phenoxyisopropyl penicillins were kindly supplied by Eli Lilly and Co., Indianapolis, Ind., as compounds 38389 and 33332, respectively. Chas. Pfizer & Co., Brooklyn, N.Y., supplied phenoxyisobutyl penicillin (compound PA-253). P-12 was supplied by Bristol Laboratories, Syracuse, N.Y., as 5-methyl-3-phenyl-4-isoxalolyl penicillin (Prostaphlin). SKF 12141 was obtained from the Smith, Kline and French Laboratories, Philadelphia, Pa. The chemical structures of the phenoxy penicillins are compared in Fig. 1.

## Results

*In vitro activity of phenoxy penicillins.* Minimal inhibitory concentrations (MIC's) of penicillin G-susceptible bacteria against six penicillins, using averages of at least 10 strains for each species, are summarized in Table 1. A serial dilution test was employed, adding 0.5 ml of a $10^{-2}$ dilution of an overnight culture to each of a series of tubes containing appropriate concentrations of antibiotic in 0.5 ml of broth. Although it is not absorbed well when administered orally, methicillin was included in this in vitro comparison to indicate its relative activity on a weight basis. The order of activity

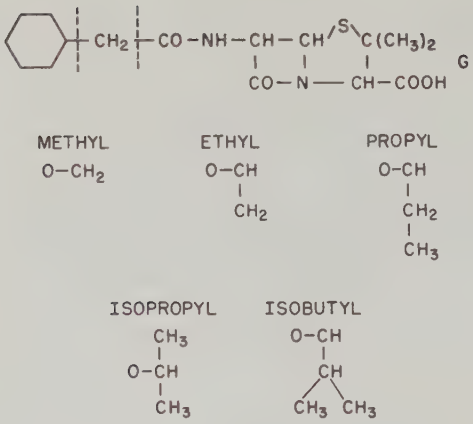


FIG. 1. *Chemical structures of phenoxy penicillins. Vertical broken lines indicate site of substitution in the penicillin G molecule.*

of the compounds, as arranged in Table 1, was found to increase from left to right, except that the isobutyl and isopropyl were reversed. Increasing the complexity of the side chain in this manner was obviously not advantageous from the standpoint of increasing antibacterial activity against nonpenicillinase-producing gram-positive bacteria. Methicillin was less active than any of the phenoxy compounds.

The percentage of strains of penicillinase-producing staphylococci, inhibited by 5 μg/ml of the same six penicillins, is presented (Table 2). Average MIC's of 25 strains, with three inoculum sizes,

TABLE 1. *Comparative activity of six penicillins against susceptible pathogens*

| Organisms | Minimal inhibitory concentration in μg/ml of penicillins | | | | | |
|---|---|---|---|---|---|---|
| | Phenoxymethyl | Phenoxyethyl | Phenoxy-propyl | Phenoxy-isopropyl | Phenoxy-isobutyl | Methicillin |
| Group A streptococci | 0.01 | 0.02 | 0.03 | 0.1 | 0.05 | 0.16 |
| Pneumococci | 0.01 | 0.02 | 0.03 | 0.1 | 0.05 | 0.16 |
| Susceptible staphylococci | 0.04 | 0.08 | 0.12 | 0.5 | 0.5 | 2.5 |

TABLE 2. *Percentage of penicillinase-producing staphylococci susceptible to six penicillins with varying inoculum sizes*

| Inoculum size (no. bacteria per ml) | Penicillins* | | | | | |
|---|---|---|---|---|---|---|
| | Phenoxymethyl | Phenoxyethyl | Phenoxy-propyl | Phenoxy-isopropyl | Phenoxy-isobutyl | Methicillin |
| | % | % | % | % | % | % |
| $10^6$ | 0 | 0 | 4 | 8 | 16 | 100 |
| $10^5$ | 0 | 5 | 10 | 50 | 100 | 100 |
| $10^4$ | 50 | 90 | 95 | 100 | 100 | 100 |

*Penicillins all are in 5 μg ml concentrations.

were used to calculate the percentages. Here, the order of activity was the opposite of that obtained with susceptible organisms, and proceeded from right to left. With the largest inoculum size, less than 20% of the strains were inhibited by the most potent drug of the phenoxy series, the isobutyl, although methicillin was 100% effective. More favorable results were obtained with smaller inoculum sizes. This was particularly impressive with the isobutyl analogue where a 10-fold decrease in the number of organisms tested resulted in inhibition of 100% of the strains.

*Serum concentrations.* Six volunteers took oral, 0.5-g doses of the above penicillins, except for methicillin, after an overnight fast, and average serum concentrations (Fig. 2) were determined by a tube-dilution method using a group A *Streptococcus* as the test organism (Roberts et al., 1961*b*). The propyl and isopropyl derivatives were better absorbed than the methyl and ethyl, but levels obtained with the isobutyl analogue were, unfortunately, no better than with the methyl.

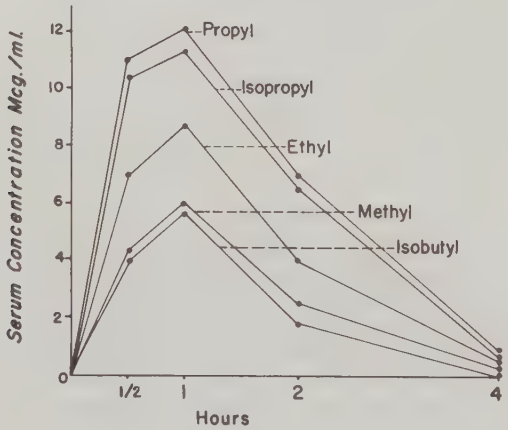


FIG. 2. *Comparative absorption of phenoxy penicillins after ingestion of a single 5-g dose.*

*Clinical trial.* Phenoxyisobutyl penicillin was administered orally to 16 patients with soft-tissue infections for 2 to 16 days. Of these, 12 received 1 g four times daily, and four were treated with half this dose. Clinical improvement occurred in all 16, but the extent to which therapy altered the course of the disease was difficult to judge due to modifications by rest, hot soaks, and surgical drainage. Three relapses occurred when therapy was stopped, a frequent occurrence with other antibiotics; 11 patients complained of bad taste of the medication; 10 had nausea; three patients had

vomiting which required discontinuing the drug in two; and two others discontinued the capsules because of diarrhea. This high incidence of gastrointestinal side effects, plus the relatively poor absorption, led us to discontinue the clinical trial.

*In vitro activity of penicillin P-12 and SKF 12141.* Average MIC's of penicillinase-producing staphylococci (77 strains) with P-12 and SKF 12141, using a large inoculum ($10^7$), are presented in Table 3. Results obtained earlier with methicillin (25 strains each of susceptible and resistant staphylococci) are included for comparison. P-12 was slightly more active than SKF 12141, particularly against penicillinase producers, and both of these compounds were much more active than methicillin. It was of interest that the relative difference between MIC's of susceptible and resistant staphylococci was greater with P-12 and SKF 12141 (more than twofold) than with methicillin (less than 50%). With group A streptococci, the average MIC's (10 strains) were: for P-12, 0.05 μg/ml; for SKF 12141, 0.07 μg/ml; and for methicillin, 0.15 μg/ml.

TABLE 3. *Average minimal inhibitory concentrations of staphylococci obtained with three penicillins*

| Strains | MIC | | |
|---|---|---|---|
| | Methicillin | P-12 | SKF 12141 |
| | *μg/ml* | *μg/ml* | *μg ml* |
| Penicillin G susceptible | 2.3 | 0.26 | 0.27 |
| Penicillinase producers | 3.8 | 0.88 | 1.1 |

*Serum binding.* Serum binding was determined by ultrafiltration of a known amount of antibiotic in 50% human serum in broth through a cellophane membrane. Air, under pressure, forced sufficient fluid for assay through the membrane within 3 hr. The broth containing 50% serum was adjusted to a pH of 7.0 prior to assay. The amounts of antibiotic, bound by serum, as determined by this method were as follows: methicillin, approximately 20%; phenethicillin, 60%; P-12 and SKF 12141, 85%.

The relatively large amount of P-12 and SKF 12141 bound by serum was also indicated by the higher MIC's obtained with these antibiotics in 50% serum as compared with broth. With P-12, for example, the average MIC of 10 penicillinase-producing staphylococci in 50% serum was 2.8 μg/ml, whereas it was only 0.8 μg/ml in broth. With a group A *Streptococcus*, representative MIC's in 50% serum and in broth were 0.26 and 0.033 μg/ml, respectively. The smaller serum to broth ratios, noted with staphylococci, were thought to be due to the unusually large inoculum, which presumably contained a high amount of protein. In contrast to these findings, MIC's with methicillin were little affected by the presence of serum (Roberts, Allen, and Kirby, 1961*a*).

*Serum concentrations.* Single- and multiple-dose crossover studies were performed with 0.5-g tablets of P-12 and SKF 12141 using six volunteers, healthy men and women between the ages of 20 and 30, for each study. A tube-dilution method was used for the assays (Roberts et al., 1961*b*), and the MIC of the test organism, group A *Streptococcus* 9282, was 0.03 μg/ml for P-12 and 0.04 μg/ml for SKF 12141.

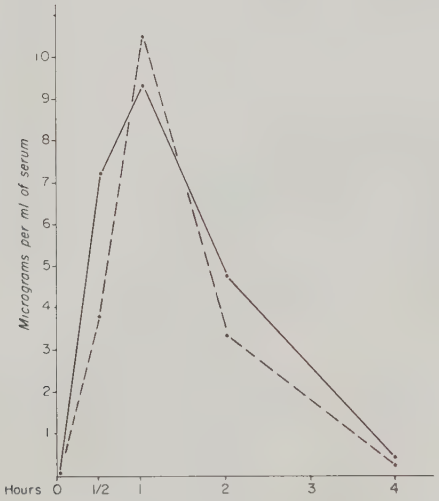


FIG. 3. *Comparative absorption of P-12 and SKF 12141 after a single, fasting, 1-g dose. Solid line is P-12 and broken line is SKF 12141.*

Average serum concentrations following ingestion of single, 1-g doses of the two drugs after an overnight fast are shown in Fig. 3. Good absorption occurred with both drugs, and significant differences were not observed in this study. Results after a 0.5-g fasting dose and again after four 0.5-g doses were taken at 4-hr intervals are shown in Fig. 4. Here, lower levels were noted with SKF 12141 on both occasions, and the levels following the fourth dose, taken 1 hr after the evening meal, were quite low with both drugs as compared with the first dose. In an attempt to minimize the effects of food, this study was repeated, giving the second and third doses 2 hr after breakfast and lunch, respectively, and the fourth dose 6 hr after lunch and 1 hr before a late dinner (Fig. 5). The levels were lower with SKF 12141 than with P-12 after each of the four doses. The 2-hr interval following breakfast and lunch was not long enough to eliminate the effects of food. With the 6-hr interval, after lunch, the concentrations of P-12 were as good as after the overnight fast.

Absorption of SKF 12141 was impaired to a greater extent by food, with the levels remaining low even after the 6-hr interval.

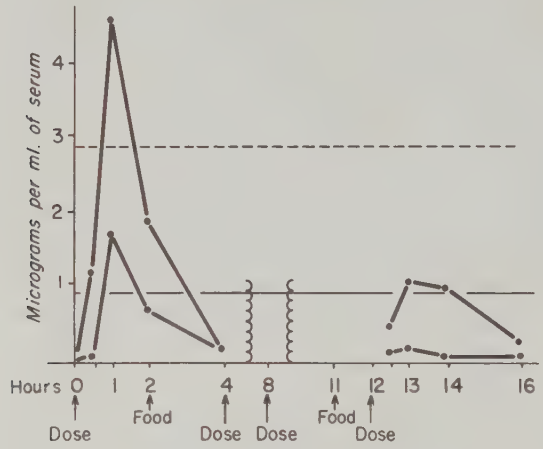


FIG. 4. *Results of a multiple-dose study showing better absorption with P-12 (upper line) than SKF 12141 (lower line).*

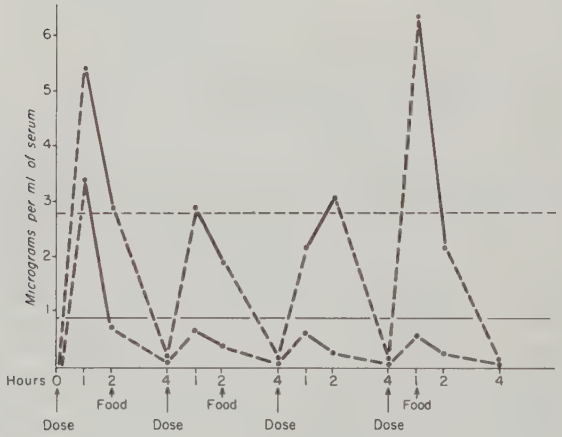


FIG. 5. *Multiple-dose study with an attempt to minimize the effects of food. P-12 (upper line) gave higher blood levels following each of the four doses than did SKF 12141 (lower line).*

## Discussion

Two general types of alteration of the chemical structure of the penicillin molecule have been utilized in the present study in an attempt to obtain compounds that are well absorbed by the oral route and have good antistaphylococcal activity.

With the phenoxy group of penicillins, some features were improved and others were made less desirable by increasing the complexity of the molecule. Activity against susceptible pathogens was decreased by lengthening the side chain, whereas it was increased against penicillinase producers. A more random pattern occurred in regard to absorption, following oral administration. Of the three compounds potentially most useful for oral therapy of infections due to nonpenicillinase producers, alpha-phenoxypropyl penicillin is less active but offers advantages in absorption and antipenicillinase activity over penicillin V and phenethicillin. Its lower degree of activity is more than offset by its better absorption, giving slightly greater antistreptococcal serum activity than with the other two compounds. The greater resistance to destruction by penicillinase cannot at present be regarded as clinically significant, and it is difficult to conclude that alpha-phenoxypropyl penicillin is likely to be clearly superior to penicillin V and phenethicillin.

Phenoxyisobutyl penicillin was not very well absorbed and caused gastrointestinal side effects, but it was active against penicillinase-producing staphylococci, with all except the largest inoculum of bacteria tested. Clinical results were good, but were difficult to assess in the soft-tissue infections treated. The degree of resistance to destruction by penicillinase necessary to insure a clinical response is not known, and there is obviously a need for establishing more precise correlations between in vitro activity and predictions of clinical efficacy. It is possible that further lengthening of the side chain to give compounds even more complex than phenoxyisobutyl penicillin will be useful, but difficulties with absorption and side effects may negate any increased resistance to penicillinase that might be achieved.

Penicillin P-12 and SKF 12141 are more similar to methicillin, in structure and in resistance to destruction by penicillinase, than are any of the phenoxy compounds. These drugs are five to eight times as active as methicillin in broth and have considerably higher MIC's in the presence of 50% serum. The clinical significance of this reversible protein binding is difficult to evaluate. However, the lower MIC of P-12 in 50% serum against resistant staphylococci as compared with methicillin in broth (2.8 versus 3.8 $\mu$g/ml) indicates that even if serum binding means complete inactivation, which is doubtful, the therapeutic effect of P-12 should be good. P-12 was slightly more active in vitro than SKF 12141 and was considerably better absorbed, particularly with 0.5-g doses given at 4-hr intervals.

Food seemed to interfere with the absorption of SKF 12141 more than with P-12. Serum concentrations following 1-g fasting oral doses of P-12 were approximately three-fourths as high as with the intramuscular injection of 1 g of methicillin (Roberts et al., 1961*a*). These in vitro and pharmacological studies indicate that P-12 administered orally should be effective in the therapy of infections due to penicillinase-producing staphylococci. Our clinical results obtained in more than 50 patients have been good, and will be reported in detail elsewhere. With SKF 12141, too few patients have been treated to allow us to draw conclusions about its therapeutic efficiency.

## Literature Cited

Roberts, C. E., Jr., J. D. Allen, W. M. M. Kirby. 1961*a*. Laboratory and clinical studies of penicillin X-1497. Clin. Pharm. Therap. 2:70-79.

Roberts, C. E., Jr., D. M. Perry, H. A. Kuharic, and W. M. M. Kirby. 1961*b*. Demethylchlortetracycline and tetracycline: A critical comparison; in vitro activity, serum concentrations and effect of serum binding. A. M. A. Arch. Internal Med. 107:204-211.

# UNTOWARD SIDE EFFECTS ASSOCIATED WITH METHICILLIN THERAPY

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Certain facts concerning untoward reactions associated with the administration of penicillin may be summarized as follows. (i) The frequency of these reactions has probably increased over the years and become more common in patients having repeated courses of penicillin. (ii) Some patients who have delayed reactions may tolerate further penicillin therapy whereas others may not. (iii) The reactions may be confined to the site of injection or may present systemic manifestations. In systemic reactions, an immediate anaphylactoid response may occur, or delayed side effects, consisting of fever, arthralgias, pulmonary infiltrates, angitis, various skin lesions, various purpuric manifestations, and nephritis, may be observed. (iv) Skin tests and serological methods are extremely unreliable in detecting the patients likely to manifest these side effects. (v) Hypersensitivity has been invoked as the usual mechanism for explaining these phenomena (Anderson, 1947; Brown, Simpson, and Price, 1959).

Bleeding of varying severity has been recognized in the skin, gastrointestinal tract, upper and lower respiratory tracts, urinary tract, and female genital tract in decreasing order of frequency. Renal abnormalities, related to penicillin hypersensitivity, have been recorded only three times in English medical literature (Spring, 1951; Unger and Nemuth, 1958; Warwick, 1960).

## Experimental

Among 92 patients treated with methicillin, six patients presented a history of previous penicillin G hypersensitivity and received methicillin for 28 days without evidence of side effects (Hewitt, Monzon, and Finegold, 1961). One patient with subacute bacterial endocarditis had a severe anaphylactic reaction after the first dose of penicillin G but, after cautious institution of methicillin, the next day was able to receive methicillin for four weeks with clinical cure and no untoward effects. During therapy, eight patients developed skin eruptions, which conformed to the usual pattern attributed to penicillin G hypersensitivity.

In several patients under therapy with methicillin, clinical phenomena suggesting hypersensitivity to methicillin or its metabolic products have been observed. Retrospective analysis of case material does not provide an entirely satisfactory estimate of the statistical frequency of phenomena such as fever, eosinophilia, and alterations in the urine and renal functions. These abnormalities were observed during the 14th to 28th day of therapy and did not occur in patients treated for less than 14 days.

The purpose of presenting the following three case histories is, first, to stimulate comment regarding the relation of side effects to hypersensitivity; second, to inquire for similar observations by other investigators; and, third, to prompt careful documentation of laboratory abnormalities during the use of 6-aminopenicillanic acid derivatives, even in the absence of clinical abnormalities.

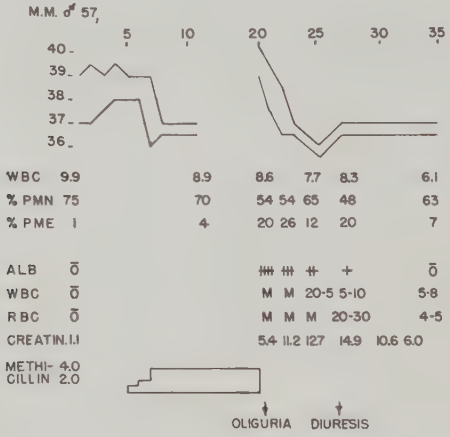


FIG. 2. *Subacute bacterial endocarditis.*

*Case 1. (M. M.). Diagnosis: Subacute bacterial endocarditis.* A 57-year-old white male (Fig. 1) was admitted with six-weeks-old subacute bacterial endocarditis which followed dental prophylaxis. Skin rash and edema of the tongue and face followed an injection of penicillin in 1952. Physical examination revealed a temperature of 39 C, cardiomegaly, mitral insufficiency, a palpable spleen, and a palpable but not enlarged liver. Laboratory abnormalities consisted of anemia, increased sedimentation rate, and Viridans streptococci in eight blood cultures. Urinalysis and creatinine were normal. Prompt resolution of sepsis, without any untoward effects, followed continuous institution of methicillin, 4.0 g daily. The patient was referred back to his local physician on the 12th day of therapy. On the 18th day of therapy, fever with chills appeared and readmission

to the UCLA Medical Center on the 20th day of therapy revealed an acutely ill patient with a fever of 40 C. Physical examination was otherwise unchanged, except for mild periorbital edema. Laboratory abnormalities consisted of marked albuminuria, pyuria, hematuria, anemia, eosinophilia of 20%, and creatinine, 5.4 mg/100 ml. Urine and blood cultures were negative, and liver function tests, antistreptolysin titer, and serum proteins were normal. Methicillin was discontinued but the patient became progressively oliguric over the next three days, with the daily urine output falling to 43 ml and the creatinine rising to 14.9 mg. Serum potassium never became elevated. Temperature returned to normal during this period and on the seventh day, after discontinuance of methicillin, urinary output began to rise accompanied by a fall in creatinine. One month after the above reaction, urinalysis was negative and continued to be negative. The creatinine was still elevated to 3.3 mg, but three months thereafter it had fallen to 2.1 mg, and four months after discontinuing methicillin it was normal.

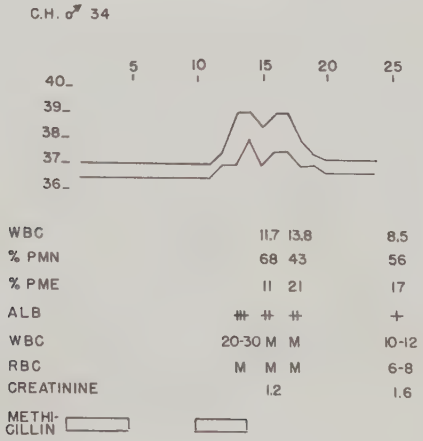


FIG. 2. *Facial pyoderma due to Staphylococcus aureus.*

*Case 2. (C. H.). Diagnosis: Facial pyoderma due to Staphylococcus aureus.* A 34-year-old white male (Fig. 2) presented multiple carbuncles of the face and severe chronic rhinitis from which *S. aureus* was cultured. When the patient was 10 years old, an ileostomy had been performed for ulcerative colitis, with no subsequent gastrointestinal difficulty. Urticarial skin eruption had followed injection of penicillin several years previously. Physical examination revealed normal vital signs, multiple, deep (1.0 to 3.0 cm), pustular lesions of the face, neck, and nares, and a well functioning right

lower quadrant ileostomy. Laboratory studies revealed normal urinalysis and blood count. Methicillin (2.0 g daily) was administered for five days with marked clearing of lesions. Methicillin was reinstituted six days later, because of recurring lesions. On the third day of therapy, fever and chills, urinary frequency, and left flank pain appeared, and on the fourth day gross hematuria was noted. Physical examination revealed a temperature of 39.2 C, a pinkish, maculopapular skin eruption over the thorax and flanks, bilateral costovertebral angle tenderness, and marked suprapubic tenderness. Laboratory abnormalities consisted of marked albuminuria, pyuria, hematuria, and leukocytosis of 11,700 with eosinophilia of 11%. Cultures of urine, blood, and antistreptolysin titer were negative. Creatinine rose from 1.2 to 1.6 mg. After discontinuance of methicillin, temperature returned to normal in five days, and in twelve days a decrease in urinary abnormalities and eosinophilia was noted. By this time the patient was symptomatically well. Urinalysis, blood count, and creatinine were normal 11 months later.

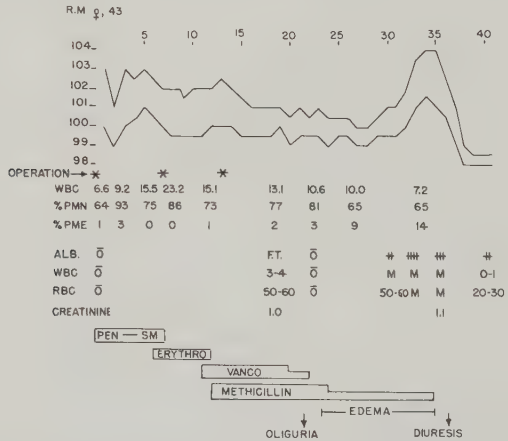


FIG. 3. *Acute mediastinitis, Staphylococcus aureus.*

*Case 3. (R. M.). Diagnosis: Acute mediastinitis, S. aureus.* A 43-year-old white female (Fig. 3) with a heart murmur for many years was admitted for elective aortic valvulotomy. An episode of pneumonia, one year earlier, had been treated with procaine penicillin for 10 days with good response and without evidence of drug hypersensitivity. Physical examination revealed cardiac enlargement and a grade 4 pansystolic aortic murmur. Urinalysis and blood count were normal. Fracture of a tiny, rigid, aortic aperture was accomplished. Fever ranged from 101 to 103 F during the immediate postoperative period while procaine penicillin with streptomycin was administered, and on the eighth postoperative day, spontaneous purulent

wound drainage appeared containing *S. aureus* in pure culture. Despite attempts at adequate drainage and administration of erythromycin, the febrile course with leukocytosis continued and left pleural effusion appeared. Vancomycin and methicillin were instituted, and on the 15th postoperative day, further debridement and dependent suction drainage were accomplished. Following these measures general clinical improvement occurred; fever and leukocytosis decreased; the appearance of the wound improved; and the left pleural fluid was demonstrated to be uninfected. On the 20th postoperative day urinary abnormalities were observed and ascribed to vancomycin. On the 23rd postoperative day, weight gain, edema, and oliguria were noted, and vancomycin was stopped. Although urinalysis at the time of edema and oliguria was recorded as normal, this finding, in view of subsequent observations, may be subject to question. These abnormalities increased, eosinophilia appeared, and on the 33rd postoperative day a sharp febrile rise occurred. The urine sediment at this time revealed many erythrocytes, leukocytes, renal tubular epithelial cells, oval fat bodies, and casts of all kinds, but especially tubular epithelial cell casts. Urine and blood cultures were negative, and the antistreptolysin titer and creatinine remained normal. Eosinophilia was also present. Following discontinuance of methicillin, the fever subsided dramatically, marked diuresis occurred, and five days later proteinuria and hematuria were decreasing with only a rare blood cell cast in the urinary sediment. Two months after discontinuance of antibiotics the patient was clinically well, and urinalysis, blood count, and creatinine were normal.

## Literature Cited

Anderson, A. B. 1947. Anaphylactic purpura following intramuscular penicillin therapy. Med. J. Australia **1**:305-306.

Brown, W. J., W. G. Simpson, and E. V. Price. 1960. Preliminary evaluation of the incidence of reaction to penicillin. Antibiotics Ann. 1959-1960, p. 516-520.

Hewitt, W. L., O. Monzon, and S. M. Finegold. 1961. Clinical experience with dimethoxyphenylpenicillin. Symposium, State University of New York, p. 127-145.

Spring, M. 1951. Purpura and nephritis after administration of procaine penicillin. J. Am. Med. Assoc. **147**:1139-1141.

Unger, A. M., and H. I. Nemuth. 1958. Penicillinase treatment of acute renal insufficiency due to penicillin hypersensitivity. J. Am. Med. Assoc. **167**:1237-1239.

Warwick, W. J. 1960. Nonthrombopenic purpura associated with penicillin hypersensitivity. Minnesota Med. **43**:484-487.

# EVALUATION OF 2,6-DIMETHOXYPHENYL PENICILLIN THERAPY IN STAPHYLOCOCCAL SEPSIS

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Within 2 years after isolation of 6-aminopenicillanic acid by Batchelor et al. (1959), a new penicillin was synthesized which has unique properties in regard to its action against penicillin G-resistant staphylococci. This antibiotic, 2,6-dimethoxyphenyl penicillin, is remarkably resistant to destruction by the penicillinase enzyme of both *Bacillus cereus* and *Staphylococcus aureus* (Douthwaite and Trafford, 1960; Gourevitch et al., 1960; Griffith et al., 1960). It thereby maintains its antibiotic effect even though capable of inducing high levels of penicillinase production (Douthwaite and Trafford, 1960; Finland, Jones, and Barnes, 1959). 2,6-Dimethoxyphenyl penicillin has been enthusiastically received by the medical profession, and early reports (Hewitt et al., 1960; Rifkind and Knight, 1960; Rolinson et al., 1960; Rutenburg, Greenberg, and Schweinburg, 1960; Steinman, 1960) have indicated excellent therapeutic results in the treatment of staphylococcal sepsis.

It is the purpose of this communication to analyze experience with 2,6-dimethoxyphenyl penicillin in 43 cases of proven coagulase-positive staphylococcal sepsis, both with regard to its clinical efficacy and potential hazards.

## Materials and Methods

A total of 196 isolates of coagulase-positive staphylococci, including those from 49 patients treated with dimethoxyphenyl penicillin, were tested for sensitivity to this antibiotic by the tube-dilution method. In addition, comparative sensitivity tests were performed on the majority of isolates with penicillin G, vancomycin, chloramphenicol, novobiocin, erythromycin, and tetracycline. Saline dilutions of dimethoxyphenyl penicillin were prepared from a dry powdered standard containing an equivalent of 18 mg of the acid monohydrate. Concentrations of the antibiotic ranged from 450 to 0.89 $\mu$g per ml.

Concentrations of penicillin G ranged from 200 units to 0.4 units per ml and for all other antibiotics, from 200 to 0.4 μg per ml. Each tube was inoculated with a $10^2$ dilution of an 18-hr Trypticase soy broth culture (pH 7.3) containing the respective *Staphylococcus*, and all tubes were examined after 24 and 48 hr of incubation at 37 C. Suitable growth controls were employed. The minimal inhibitory concentration (MIC) was recorded as the lowest concentration of antibiotic that prevented visible growth in 24 hr. The bactericidal action of dimethoxyphenyl penicillin on 83 strains was evaluated by subculturing one standard loopful from each clear tube beyond the MIC to 110 staphylococcal media (Difco).

All patients included in this study were personally studied and followed by the author. The following laboratory data were obtained both before and after treatment in all patients: hemoglobin, white cell count, differential count, urinalysis, blood urea nitrogen, and alkaline phosphatase or serum glutamic oxaloacetic transaminase.

*Treatment population.* The results of dimethoxyphenyl penicillin therapy are summarized for 43 of 54 patients who received the antibiotic. Of 11 patients not included in the study, three were patients from whom no staphylococci could be cultured, four were concurrently on another effective antibiotic, coagulase-negative staphylococci only were isolated from two, and two patients were excluded for reasons of inadequate dosage.

The age range of infected patients was fairly evenly distributed, with a slight predominance of older age groups. Four of seven infections occurring within the first decade followed chemotherapy of acute lymphatic leukemia. In older age groups, infections following surgery comprised 61% of that population. Most patients were extremely ill as the result of major surgery or underlying disease and usually were not referred for dimethoxyphenyl penicillin therapy until other antibiotics had failed. These features may have contributed to an over-all mortality of 39.5%.

The age range of those treated was 3 weeks to 81 years with an average of 41.4 years. Dimethoxyphenyl penicillin (generously supplied as Staphcillin by Bristol Laboratories, Syracuse, N.Y.) was given in a dosage of 1 g every 4 to 6 hr, either intramuscularly or intravenously. Five patients received 2 g every 4 hr during early phases of their treatment. Children received 25 mg per kg, intramuscularly every 4 hr. The average dose was 44.8 g given over 10.9 days. The largest total dose was 224 g given over 57 days to a patient with osteomyelitis.

*In vitro studies.* The resistance to antibiotics of staphylococci isolated from 49 of 54 patients receiving dimethoxyphenyl penicillin is summarized in Table 1. Resistance appears directly proportional to the amount and time through which a particular antibiotic has been used at the University of Minnesota Hospitals. Penicillin G displayed little efficacy against the staphylococci in this population,

as indicated by the fact that 44 of 46 isolates were resistant to greater than 100 units per ml by the tube-dilution method. Similarly, 37 of 46 isolates were resistant to greater than 200 μg per ml of erythromycin.

TABLE 1. *Antibiotic sensitivities using the tube-dilution method on staphylococcal isolates from 49 patients*

| Antibiotic | Concentration in μg/ml | | | | | | | | | | Total isolates |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | 200 | 100 | 50 | 25 | 12.5 | 6.3 | 3.2 | 1.6 | 0.8 | 0.4 | |
| Penicillin G* | - | 44† | 1 | - | - | - | - | - | - | 1 | 46 |
| Vancomycin | - | - | 1 | - | 2 | 12 | 12 | 1 | - | - | 28 |
| Chloromycetin | - | 14 | 12 | 3 | 10 | 7 | - | - | - | - | 46 |
| Novobiocin | - | 2 | 3 | 3 | 15 | 13 | - | - | 7 | 3 | 46 |
| Erythromycin | 37 | - | - | - | - | - | - | 1 | 5 | 3 | 46 |
| Tetracycline | - | - | - | 5 | 19 | 2 | 1 | - | 3 | - | 30 |

*Measured in units/ml.
†> 100.

Tests of sensitivity to dimethoxyphenyl penicillin were performed by the tube-dilution method on 196 staphylococcal isolates, including those from the 43 patients under discussion. Isolates sensitive to the antibiotic (in μg/ml) are as follows: no isolates, 0.9; five isolates, 1.8; 116 isolates, 3.5; 71 isolates, 7.0; four isolates, 14.0; and no isolates, 28.1. Total bactericidal end points were not obtained in 83 isolates randomly selected from this group (Fig. 1). During the first 72 hr, after exposure of a $10^2$ dilution of an 18-hr culture to dimethoxyphenyl penicillin, growth was obtained even from tubes containing as much as 225 μg per ml. Scanty growth of inocula taken from tubes above the MIC at 120 or 144 hr is probably related to senescence and death of persisting organisms, rather than to any effect of the antibiotic which is largely inactivated after 1 week at 37 C (Stewart and Harrison, 1960).

## Results

*Definitions of response to dimethoxyphenyl penicillin.* To carefully define clinical response to therapy, the following criteria were outlined: elimination of all *S. aureus* as determined by negative culture or complete recovery, *excellent;* apparent healing of staphylococcal lesions or eradication of septicemia associated therewith, as proven by negative blood culture for *S. aureus* before death or at autopsy, *improved;* and persistence of staphylococci, proved by culture at death or on completion of dimethoxyphenyl penicillin therapy, *failure*.

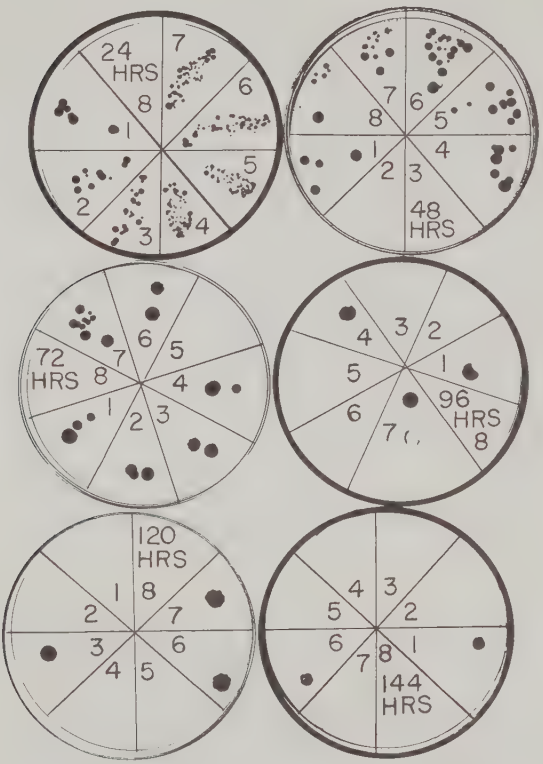


FIG. 1. *Photograph of serial 24-hr inoculations from a representative isolate of Staphylococcus with an MIC of 3.5 μg/ml. Antibiotic concentrations from each clear tube above the MIC are identified by consecutively numbered segments. 1 = 225 μg/ml, 2 = 112.5 μg/ml, 3 = 56.2 μg/ml, 4 = 28.1 μg/ml, 5 = 14.0 μg/ml, 6 = 7.0 μg/ml, 7 = 3.5 μg/ml, 8 = blank. A second streaking of the dilution contained in sector 7 is seen in sector 8 of the 48-hr plate.*

Particular emphasis is placed on the "improved" category because several patients died of other metabolic or organic complications at a time when their staphylococcal problem was well on the way to resolution.

In reducing the clinico-pathologic complexities of this population to a common denominator which could be scaled in terms of the preceding definitions, all infections were classified as to anatomic

site, these being corium, deep tissue, blood (septicemia), deep tissue and blood, urinary tract, and nose. Regardless of their location, 60.5% of all infections were completely eradicated (Table 2). Definite improvement was shown in 16.3%, and 23.3% were treatment failures. Thus, 76.8% of all patients were either cured of staphylococcal sepsis or definitely improved as defined under the above conditions. The excellent response of patients with combined involvement of deep tissue and blood is explained in part by the striking nature of their symptoms, thereby attracting vigorous medical therapy at an earlier stage than some of the deep loculated tissue infections.

TABLE 2. *Location and response to dimethoxyphenyl penicillin of 43 staphylococcal infections*

| Location | Excellent* | Improved | Failure | Total |
|---|---|---|---|---|
| Corium | 2 | 1 | 1 | 4 |
| Deep tissue | 6 | 1 | 6 | 13 |
| Blood | 1 | - | 1 | 2 |
| Deep tissue + blood | 14 | 5 | 2 | 21 |
| Urinary tract | 2 | - | - | 2 |
| Nose | 1 | - | - | 1 |
| Total | 26 | 7 | 10 | 43 |
| Percentage | 60.5 | 16.3 | 23.2 | |

*Terms defined in Results section.

Pathological findings, manifested by nine of 10 failures under dimethoxyphenyl penicillin therapy, are as follows. In two patients with acute lymphatic leukemia, the infections were of the corium and the blood. Infections involving deep tissue and blood combined occurred in two patients, one having acromegaly, renal failure, and staphylococcal endocarditis, and another with rectal carcinoma perforating his flank and having only 36 hr of treatment. In five cases with deep tissue infections, the complications were: pyo-arthritis, terminal lymphosarcoma in one; terminal hypernephroma with gluteal abscess in another; and in the three remaining postcardiotomy patients, one had a mediastinal abscess with an aortic perforation and two had retrosternal abscesses. The particularly poor defense mechanism of these patients serves to re-emphasize the futility of any antibiotic therapy in such cases. The two postcardiotomy patients with retrosternal abscesses recovered completely after excision of all necrotic tissue, thus reaffirming the need for adequate surgical drainage of all loculated infections.

*Adverse reactions.* Side reactions occurring in conjunction with dimethoxyphenyl penicillin therapy were seen in four of the 54 patients treated. Two cases of dermatitis medicamentosa were encountered. The first was characterized by a transient macular-erythematous rash which developed after 36 hr of therapy and

disappeared rapidly when the dosage was reduced from 1 g every 4 hr to 1 g every 6 hr, intramuscularly. This patient had received penicillin V and potassium iodide without incident prior to initiation of dimethoxyphenyl penicillin and had no history of allergy to either drug. The second case of dermatitis was more severe, with confluent macular-erythematous areas developing over much of the body on the 17th day of therapy. This rash slowly disappeared several days after the drug was stopped. The patient also received tetracycline concurrently but had shown no hypersensitivity to this antibiotic or penicillin G on prior exposure.

An interesting example of immediate dermal sensitivity was encountered in a physician who used a nasal spray of dimethoxyphenyl penicillin to eradicate a staphylococcal nasal carrier state. With his first contact, the patient suffered an immediate rhinitis which progressed to intense erythema and vesiculation of the face during the ensuing 48 hr of spraying twice daily. All symptoms resolved 72 hr after spraying was stopped. He had acquired an exquisite cutaneous sensitivity to penicillin G while working as a pipe fitter at a penicillin production center in 1951. In 1953 he exfoliated following one injection of penicillin G.

A more serious reaction occurred in a 31-year-old male who developed a retrosternal abscess following cardiotomy for repair of

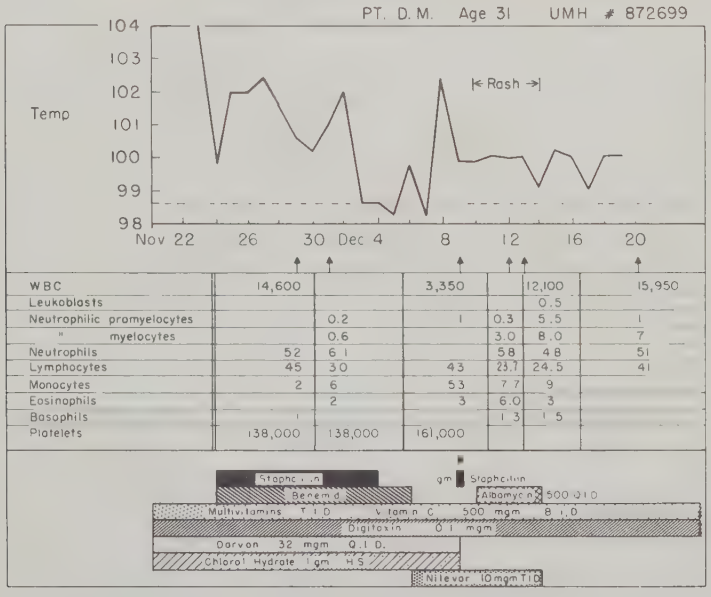


FIG. 2. *Clinical course of a 31-year-old male who developed transient agranulocytosis of peripheral blood in association with dimethoxyphenyl penicillin therapy.*

an atrioventricular communis defect (Fig. 2). He improved steadily on dimethoxyphenyl penicillin, but 5 days after initiation of therapy, petechial hemorrhages were noted following application of a tourniquet for venipuncture. Antibiotic therapy was continued another 5 days without noticeable effect on his haemopoetic system. Four days later his condition deteriorated and his temperature rose sharply to 102.4 F. A differential count done on peripheral blood at this time revealed almost complete agranulocytosis with 1 neutrophil promyelocyte, 43 lymphocytes, 53 monocytes, and 3 eosinophils. An order to resume dimethoxyphenyl penicillin, because of the fever spike, was canceled after a 1-g dose had been given. Within 72 hr, neutrophils reappeared in his peripheral blood, and a bone marrow examination done at this time was regarded as nondiagnostic. All other medications which the patient had received, with the exception of probenecid, have been given subsequently without ill effect. Patch and intradermal tests with dimethoxyphenyl penicillin were negative. Although absolute certainty is difficult to establish, this case strongly suggests an instance of hypersensitivity to dimethoxyphenyl penicillin resulting in transient agranulocytosis.

No instances of hepatic or renal toxicity, which could be attributed to dimethoxyphenyl penicillin, were noted in this series.

## Discussion

Under the conditions of this study, dimethoxyphenyl penicillin appears to be a major therapeutic addition to the treatment of sepsis caused by penicillin G-resistant staphylococci.

The appearance of four superinfections by gram-negative organisms in this series emphasizes the importance of secondary invaders in unhealed areas of recent primary infection. In view of the increasing seriousness of nosocomial mixed infections (Stewart, Nixon, and Coles, 1960), combined treatment with dimethoxyphenyl penicillin and a broad-spectrum antibiotic is to be recommended in infections where gram-negative organisms are present in addition to staphylococci.

A note of caution is suggested by the in vitro observation that dimethoxyphenyl penicillin is not completely bactericidal to staphylococci when large inocula are used. This is explained by the fact that large inocula contain slowly metabolizing organisms which have reached a stationary phase of growth and are resistant to antibiotics, which are most effective during the log phase of bacterial growth. Yow (1960) has offered supportive in vivo evidence, demonstrating persistence of potentially lethal staphylococci in abscesses of experimental animals, despite high concentrations of antibiotics within these sites maintained by parenteral injection. An analogous situation arose in one of our patients (Fig. 3) who developed a staphylococcal

septicemia and wound infection following partial nephrectomy for removal of a stag-horn calculus. He responded well to dimethoxyphenyl penicillin through 20 days of therapy (88 g). But, 20 days after cessation of therapy he suddenly developed left heart failure and died 13 days later of staphylococcal endocarditis which had perforated his aortic valve in two places. This type of case suggests that where deep abscess formation may have arisen in conjunction with staphylococcal septicemia, properly selected antibiotic therapy should be employed in large doses over a prolonged period. Only by such continuous antibiosis can one hope to eradicate organisms whose anatomical location or inactive metabolism make them unassailable by less vigorous antibiotic regimens.

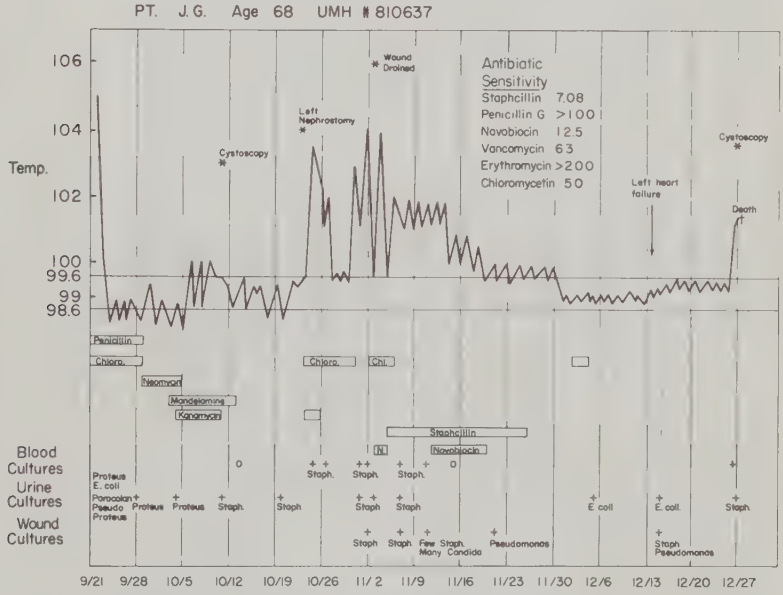


FIG. 3. *Clinical course of a 68-year-old male who died of staphylococcal endocarditis following 20 days of therapy with dimethoxyphenyl penicillin.*

A 7% incidence of side reactions to dimethoxyphenyl penicillin in our series illustrates the sensitizing properties of this drug. Despite preliminary evidence that penicillin G and dimethoxyphenyl penicillin are immunologically distinct (Rifkind and Knight, 1960), the latter should be given with caution to those with known penicillin G allergy or whose history is otherwise marked by the accident of allergy.

## Acknowledgments

The author wishes to express his gratitude to Wesley W. Spink for his constant interest and helpful advice during the course of this investigation. A word of thanks is also due to the University of Minnesota Hospitals medical staff for their close cooperation in this study.

## Literature Cited

Batchelor, F. R., F. P. Doyle, J. H. C. Naylor, and G. N. Rolinson. 1959. Synthesis of penicillin: 6-aminopenicillanic acid in penicillin fermentations. Nature **183**:257-258.

Douthwaite, A. H., and J. A. P. Trafford. 1960. A new synthetic penicillin. Brit. Med. J. **2**: 687-690.

Finland, M., W. F. Jones, Jr., and M. W. Barnes. 1959. Occurrence of serious bacterial infections since introduction of antibacterial agents. J. Am. Med. Assoc. **170**:2188-2197.

Gourevitch, A., G. A. Hunt, T. A. Pursiano, A. J. Moses, and J. Lein. 1960. Microbiological studies on dimethoxyphenyl penicillin, p. 4-15. *In* P. A. Bunn, [ed.], A symposium on the new dimethoxyphenyl penicillin. State University of New York, Syracuse.

Griffith, L. J., W. E. Ostrander, Z. F. Smith, and D. E. Beswick. 1960. A comparison of the reactivity, keeping characteristics and action on Staphylococcus aureus of penicillin G, phenethicillin, and dimethoxyphenyl penicillin, p. 37-42. *In* P. A. Bunn, [ed.], A symposium on the new dimethoxyphenyl penicillin. State University of New York, Syracuse.

Hewitt, W. L., O. Monzon, H. G. Dangerfield, B. Blackman, Z. Kudinoff, and S. M. Finegold. 1960. Clinical experience with dimethoxyphenyl penicillin, p. 127-135. *In* P. A. Bunn, [ed.], A symposium on the new dimethoxyphenyl penicillin. State University of New York, Syracuse.

Rifkind, D., and V. Knight. 1960. Treatment of staphylococcal and streptococcal infections with dimethoxyphenyl penicillin, p. 160-171. *In* P. A. Bunn, [ed.], A symposium on the new dimethoxyphenyl penicillin. State University of New York, Syracuse.

Rolinson, G. N., F. R. Batchelor, S. Stevens, J. C. Wood, and E. B. Chain. 1960. Bacteriological studies on a new penicillin - BRL.1241. Lancet **2**:564-567.

Rutenberg, A. M., H. L. Greenberg, and F. B. Schweinburg. 1960. Clinical experiences with 2,6-dimethoxyphenyl penicillin monohydrate in staphylococcal infections. New Engl. J. Med. **263**:1174-1178.

Steinman, H. G. 1960. Biochemical studies on 6-aminopenicillanic acid, benzyl penicillin, and 2, 6-dimethoxyphenyl penicillin, p. 17-25. *In* P. A. Bunn, [ed.], A symposium on the new dimethoxyphenyl penicillin. State University of New York, Syracuse.

Stewart, G. T., and P. M. Harrison. 1960. Microbiological studies on sodium 6-2, 6 dimethocybenzmido penicillanate monohydrate (BRL 1241) in vitro and in patients. Brit. Med. J. **2**: 694-699.

Stewart, G. T., H. H. Nixon, and H. M. T. Coles. 1960. Report of clinical use of BRL 1241 in children with staphylococcal and streptococcal infections. Brit. Med. J. **2**:703-706.

Yow, E. M. 1960. *In* P. A. Bunn, [ed.], A symposium on the new dimethoxyphenyl penicillin. State University of New York, Syracuse. Discussion, p. 123.

# CLINICAL EXPERIENCE WITH 2,6-DIMETHOXYPHENYL PENICILLIN IN THE TREATMENT OF STAPHYLOCOCCAL SEPTICEMIA

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The pathology and pathogenicity of staphylococcal septicemia have been well documented. As a complication of infection, it represents a major problem in morbidity and mortality. In the pre-antibiotic era, the mortality of staphylococcal septicemia ranged as high as 82% and the few survivors were usually patients under 40 years of age. Specific serum therapy and sulfonamides were ineffective. With penicillin and streptomycin, initially the mortality of staphylococcal septicemia steadily decreased, reaching a low of about 19% in 1947. Since then there has been a gradual increase in the mortality rate, currently estimated at about 40% (Finland, 1960).

The use of tetracyclines, erythromycin, chloramphenicol, and novobiocin in addition to penicillin and streptomycin has not appreciably altered this upward trend, primarily because of the emergence of resistant staphylococcal strains, most of which were coagulase positive and rich penicillinase producers (Spink, 1956). The predominance of these resistant strains stimulated a search for newer antibiotics resistant to degradation by penicillinase. The development of semisynthetic penicillins produced by the acylation of 6-aminopenicillanic acid (Sheehan, Henery-Logan, and Johnson, 1953; Sheehan and Ferris, 1959; Sheehan and Henery-Logan, 1959; Batchelor et al., 1959; Rolinson et al., 1960; Perron et al., 1960) has yielded antimicrobial agents effective in the control of staphylococcal disease. Of these, methicillin and more recently 5 methyl-3-phenyl-4-isoxazolyl penicillin (sodium oxacillin) were found to be resistant to hydrolysis by high concentrations of penicillinase (Rutenburg, Greenberg, and Schweinburg, 1960; Conference on 5-methyl-3-phenyl-4-isoxazolyl penicillin, 1961) and to have a bactericidal effect against resistant staphylococci. Methicillin and sodium oxacillin were also effective clinically

as well as in vitro against penicillinase-producing strains. Against strains of staphylococci which did not generate penicillinase, penicillin G was 10 to 50 times more effective on a weight basis than either methicillin or sodium oxacillin.

In a series of 230 consecutive patients, with a variety of infections due to resistant strains of staphylococci, the same microorganism was cultured from the blood stream in 11 patients. The present report deals with these 11 patients.

## Experimental

*Bacteriological findings.* *Staphylococcus aureus*, coagulase positive and resistant to penicillin G, was isolated from all 11 patients. In vitro sensitivity studies of methicillin and sodium oxacillin, using a serial tube-dilution method (Schweinburg, Sylvester, and Rutenburg, 1954), indicated that the bactericidal titer of these strains ranged from 0.18 to 3.12 μg/ml for methicillin and from 0.18 to 1.56 μg/ml for sodium oxacillin. The bactericidal titer did not vary with larger inocula. This was a measure of the resistance of these penicillins to penicillinase.

*Clinical observations.* The ages ranged from 34 to 78 years with the majority of patients between 55 to 65 years; six were males and five were females (Table 1). In three patients concurrent steroid therapy is believed to have enhanced the virulence of the infection. Three had diabetes mellitus with associated Kimmelstiel-Wilson disease. A fourth patient had chronic myelogenous leukemia for which he was also given corticosteroids, radiation, and chemotherapeutic (alkylating) agents. He developed numerous soft-tissue staphylococcal infections. The fifth patient had an allergic vasculitis. In the course of corticosteroid therapy, he developed deep soft-tissue sepsis of the shoulder, arm, and presacral area. Two patients developed staphylococcal wound infection at the site of a cutdown with subsequent brachial vein septic thrombophlebitis. The eighth patient developed acute endometritis and septicemia following an attempted abortion. The remaining three patients developed septicemia. In one, sepsis was at the site of an aortic graft, in another secondary to postoperative pneumonia, and in the third after excision of a giant nevus of the buttocks.

## Results

Methicillin or sodium oxacillin were given intramuscularly or intravenously daily in divided doses of 4 to 20 g (Table 1). In some instances, both the intravenous and the intramuscular routes were used,

TABLE 1. *Survey of 11 patients with various infections who developed staphylococcal septicemia*

| Case no. | Age | Sex | Primary diagnosis | Portal of entry | Probable precipitating factor | Prior antibiotic therapy | Effective antibiotic | Daily dose | Route | Duration of therapy | Result | Remarks |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | yr | | | | | | | g | | days | | |
| 1 | 64 | M | Diabetes mellitus, glomerulonephritis | Genito-urinary | Indwelling catheter | Penicillin G, erythromycin, chloramphenicol | Methicillin | 20<br>12<br>6 | iv<br>im<br>im | 2<br>7<br>25 | Good | Slow defervescence and sterilization of blood |
| 2 | 65 | F | Diabetes mellitus, simple mastectomy for cystic disease | ?Skin<br>?Wound | Wound infection | Telomycin | Methicillin | 10<br>6 | im<br>im | 1<br>4 | Good | Rapid defervescence and clearance of wound |
| 3 | 67 | M | Diabetes mellitus | Skin | Carbuncle | Penicillin G, streptomycin, tetracycline, chloramphenicol | Methicillin | 16<br>6 | im<br>iv<br>im | 6<br>14 | Good | Defervescence and healing after methicillin and surgical excision |
| 4 | 34 | M | Chronic myelogenous leukemia | Skin | Steroid therapy, radiation, alkylating and chemotherapeutic agents | – | Methicillin | 6<br>4 | iv<br>im | 1<br>21 | Good | Defervescence and sterilization of wounds after methicillin and surgical excision |
| 5 | 61 | M | Allergic vasculitis, uremia, soft tissue sepsis | Skin | Prolonged bed rest, decubitus ulcers, steroid therapy | – | Methicillin | 14<br>8<br>4 | im<br>im<br>im | 1<br>4<br>20 | Good | Control of sepsis by surgical excision of septic foci and methicillin |
| 6 | 61 | M | Chronic anemia, unclassified blood dyscrasia, urinary tract infection, wound infection, septic phlebitis, multiple carbuncles, acute endocarditis | Skin<br>Wound<br>Urinary tract | Cardiac catheterization, steroid therapy | Chloramphenicol erythromycin | Methicillin | 4<br>6<br>4-6 | im<br>iv, im<br>im | 4<br>2<br>6 | Failure | Failure to control infection resulted in acute endocarditis, ?inadequate methicillin therapy |
| 7 | 55 | F | Multiple furnuncles, septic wound, septic phlebitis | Skin | Infected cutdown | – | Methicillin | 6<br>4 | im<br>im | 1<br>9 | Good | Defervescence and healing |
| 8 | 35 | F | Endometritis | Uterus | Abortion | – | Methicillin | 6<br>4 | im<br>im | 1<br>6 | Good | Control of sepsis with methicillin, followed by dilatation and curettage |
| 9 | 79 | F | Pneumonia after colectomy | Respiratory system | Postoperative anesthesia | Penicillin G, novobiocin | Methicillin | 6<br>4 | im<br>im | 2<br>8 | Good | Rapid clearance |
| 10 | 60 | M | Septic aortic graft | ?Vascular<br>?Wound<br>?Skin | ?Foreign body | Penicillin G, streptomycin tetracycline | MPI penicillin | 3<br>3<br>4 | im<br>ip<br>im | 10<br>7<br>7 | Failure | Failure to sterilize aortic graft |
| 11 | 50 | F | Excision of nevus of buttocks | Skin | Wound infection | Tetracycline, kanamycin, penicillin G | Methicillin | 6<br>4 | im<br>im | 1<br>10 | Good | Defervescence and healing |

and one patient treated with sodium oxacillin received the drug intraperitoneally as well. Where indicated, surgical drainage, wound debridement, or removal of foreign bodies was carried out. The duration of therapy ranged from 5 to 34 days and the total dose of methicillin from 30 to 257 g.

Nine of the 11 patients survived. One of the two patients who died had chronic anemia due to an unclassified blood dyscrasia, requiring corticosteroid therapy, and a staphylococcal urinary tract infection. He developed an infected phlebotomy incision, septic phlebitis extending into the brachial vein, and staphylococcal septicemia. After the initial course of methicillin he improved and became asymptomatic. Blood cultures became sterile and he was discharged. A recurrence of staphylococcal wound infection, multiple carbuncles, and septicemia required readmission. He failed to respond to three courses of methicillin of 4, 2, and 6 days duration, respectively, developed acute endocarditis, and died. The responsible microorganism, a coagulase-positive *S. aureus*, remained sensitive to methicillin with a minimal inhibitory concentration (MIC) of 0.78 μg/ml. Factors responsible for therapeutic failure in this patient included inadequate methicillin therapy; corticosteroid therapy; and anemia, blood dyscrasia, and a chronic state of debilitation and impaired host resistance.

The second patient developed intermittent fever and leukocytosis 9 days after resection of an aortic aneurysm and dacron graft (Fig. 1). He was explored because of sudden vascular collapse. At operation he had extensive periaortic and perigraft sepsis with septic intra-aortic thrombosis. Cultures of the peritoneal exudate, aorta, aortic thrombus, and peripheral blood yielded coagulase-positive, penicillin G-resistant *S. aureus*, which was sensitive to sodium oxacillin

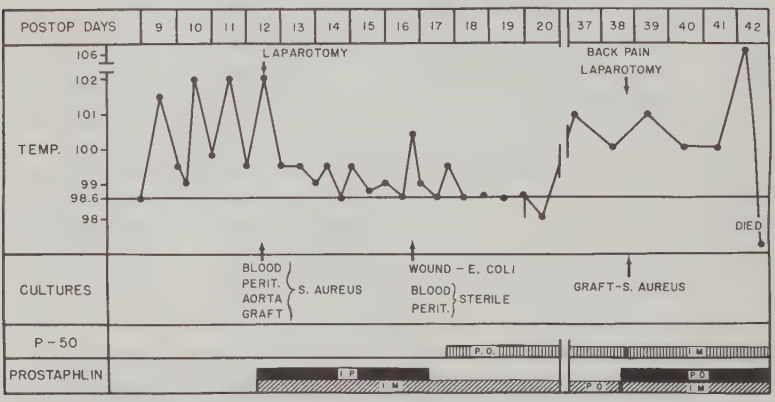


FIG. 1. *Therapy in a 60-year-old male with infected aortic graft and staphylococcal septicemia.*

(MIC, 0.18 $\mu$g/ml). Postoperatively he received sodium oxacillin daily intramuscularly (3 g) and intraperitoneally (1.5 g). His recovery was remarkably prompt and progressive with sterilization of the blood and peritoneal fluid. A wound infection due to *Escherichia coli* was controlled with $\alpha$-amino benzyl penicillin (2 to 4 g daily). He was well until the 37th day after the exploratory laparotomy when he developed severe back pain. Exploration revealed leakage at the aortic suture line with a huge retroperitoneal hematoma. The graft was replaced with considerable difficulty. Cultures of a segment of the old dacron graft yielded *S. aureus*. This confirmed the familiar contention that infections cannot be controlled nor wounds sterilized in the presence of a foreign body. His subsequent postoperative course was poor. He developed auricular fibrillation, vascular collapse, and died 72 hr later, probably because of myocardial infarction. At post mortem there was no evidence of infection, thrombosis, or leakage at the graft site.

## Case Reports

*Case 1.* A 64-year-old male with diabetes mellitus and glomerulonephritis after insertion of an indwelling catheter developed a staphylococcal urinary tract infection (Fig. 2). He failed to respond to penicillin G, erythromycin, and chloramphenicol. He was critically

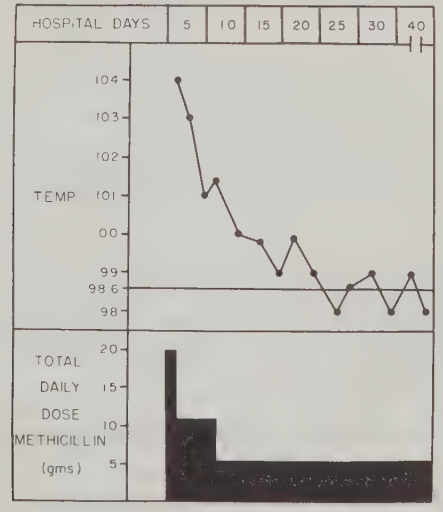


FIG. 2. *Therapy in a 64-year-old male with glomerulonephritis, diabetes mellitus, and staphylococcal septicemia.*

ill with intermittent fever to 104 F, and coagulase-positive *S. aureus* was cultured from his blood. Methicillin, 20 g/day in divided doses, was given intravenously for 2 days and 12 g/day in divided doses intramuscularly for 7 days. He showed sterilization of the blood and gradual subsidence of the signs and symptoms of his infection. Methicillin therapy was continued intramuscularly for 25 additional days with gradual decrease in the daily dose. He was cured after 34 days of treatment with a total dose of 274 g.

*Case 2.* A 34-year-old male was hospitalized with chronic myelogenous leukemia. His therapy included corticosteroids, radiation, and alkylating agents (Fig. 3). He developed extensive soft-tissue infection with multiple furuncles and carbuncles, which involved most of his body surfaces, and a septicemia due to *S. aureus* resistant to penicillin G, streptomycin, tetracycline, and chloramphenicol. He was in severe pain, febrile, and semicomatose. After 24 hr of intravenous methicillin therapy (8 g) his striking improvement and control of infection permitted appropriate surgical excision, drainage, and debridement of 62 soft-tissue lesions. After 6 days of therapy, cultures of the wounds, peripheral blood, pharynx, and skin were negative for staphylococci. He received methicillin for 3 weeks and recovered from his infection uneventfully.

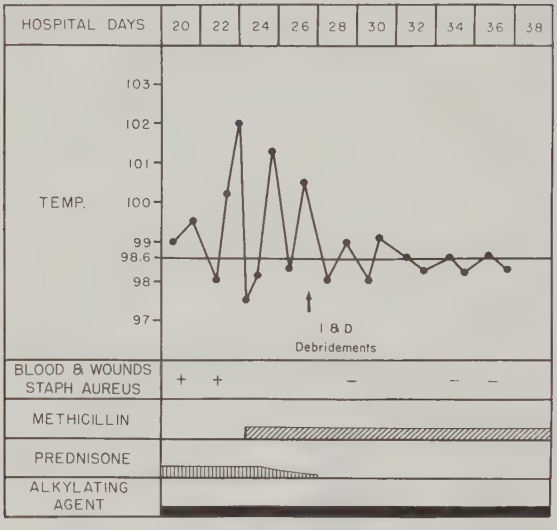


FIG. 3. *Therapy in a 34-year-old male with leukemia, multiple carbuncles, and staphylococcal septicemia.*

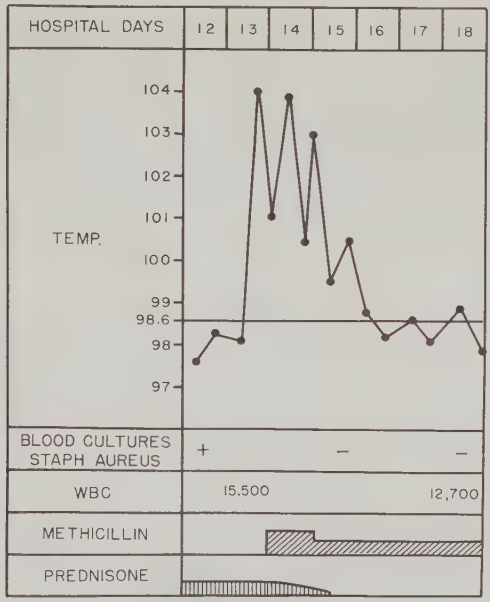


FIG. 4. *Therapy in a 61-year-old male with allergic vasculitis, soft tissue sepsis, and staphylococcal septicemia.*

*Case 3.* A 61-year-old patient had hypertension and renal failure due to allergic vasculitis (Fig. 4). His primary disease responded to corticosteroids but he developed an infection of the soft tissues of the buttocks, scapula, presacral area, and elbow. Resistant staphylococci were cultured from his wounds as well as his blood. After 24 hr of methicillin therapy (14 g), intramuscularly and intravenously, he showed defervescence and striking control of his infection. The suppurative necrotic tissue was excised and adequate drainage of all soft-tissue lesions established. Thereafter, he became afebrile and cultures of the wounds were negative for staphylococci. Methicillin was continued for 25 days during which secondary closure of the large soft-tissue defects was carried out successfully.

## Discussion

The data herein presented indicate the efficacy of methicillin in the treatment of staphylococcal septicemia. The survival of 9 of 11 patients, in many of whom the infection was compounded by underlying chronic disease, is an eloquent testimony of the efficacy of

semisynthetic penicillins in the treatment of staphylococcal sepsis. Minimal requirements of therapy are early recognition and bacteriological diagnosis, prompt and *adequate* antibiotic therapy with an effective agent, and surgical control of the primary foci of infection by drainage, debridement, and removal of foreign bodies.

No side reactions or toxic effects were observed with the administration of the required high doses for prolonged periods. As with penicillin G, the modified semisynthetic penicillins have a very low index of toxicity.

## Literature Cited

Batchelor, F. R., R. P. Doyle, H. H. C. Naylor, and G. N. Rolinson. 1959. Synthesis of penicillin: 6-aminopenicillanic acid in penicillin fermentations. Nature **183**:257-258.

Conference on 5-methyl-3-phenyl-4-isoxazolyl penicillin (Prostaphlin). 1961. Abstracts of papers. Bristol Laboratories, Syracuse.

Finland, M. 1960. Treatment of pneumonia and other serious infections. New Engl. J. Med. **263**: 207-221.

Perron, Y. G., L. B. Crast, W. J. Gottstein, W. F. Minor, and I. C. Cheney. 1960. Synthesis of potassium penicillin-152 [potassium (α-phenoxyethyl)-penicillin] and both diastereoisomers. Antibiotics Ann. 1959-1960, p. 107-110.

Rolinson, G. N., S. Stevens, F. R. Batchelor, J. C. Wood, and E. B. Chain. 1960. Bacteriological studies on a new penicillin, BRL-1241. Lancet **2**:564-567.

Rutenburg, A. M., H. L. Greenberg, and F. B. Schweinburg. 1960. Clinical experiences with 2,6 dimethoxyphenyl penicillin monohydrate in staphylococcal infections. New Engl. J. Med. **263**:1174-1178.

Schweinburg, F. B., E. M. Sylvester, and A. M. Rutenburg. 1954. Relation of in vitro sensitivity test to clinical response to Aureomycin therapy. Surgery **35**:204-212.

Sheehan, J. C., and J. P. Ferris. 1959. The chemical conversion of penicillin G into a biologically active synthetic penicillin series. J. Am. Chem. Soc. **81**:2912.

Sheehan, J. C., and K. R. Henery-Logan. 1959. A general synthesis of the penicillins. J. Am. Chem. Soc. **81**:5838-5839.

Sheehan, J. C., K. R. Henery-Logan, and D. A. Johnson. 1953. The synthesis of substituted penicillins and simpler structural analogs. VII. The cyclization of a penicilloate derivative to methyl phthalimidopenicillanate. J. Am. Chem. Soc. **75**:3292-3293.

Spink, W. W. 1956. The clinical problem of antimicrobial resistant staphylococci. Ann. N. Y. Acad. Sci. **65**:175-189.

# EFFECTIVENESS OF 2, 6-DIMETHOXYPHENYL PENICILLIN IN PATIENTS WITH CYSTIC FIBROSIS

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The control of antibiotic-resistant staphylococcal infections of the respiratory tract presents a major problem in the management of patients with cystic fibrosis (di Sant'agnese, 1956; Huang, 1957; Schwachman, 1960). The monohydrate sodium salt of 2,6-dimethoxyphenyl penicillin (DMP), a new semisynthetic penicillin, has recently been found to be resistant to penicillinase and bactericidal against *Staphylococcus aureus*. Thus, it has been investigated for its effectiveness in children with cystic fibrosis, and the study is still in progress.

## Materials and Methods

A total of 14 cystic fibrosis (CF) patients were treated with 17 courses of intramuscularly administered DMP, and two of these patients were concurrently treated by aerosol inhalation. The age, weight, dosage regimens, and duration of treatment of these patients are summarized in Table 1. The daily intramuscular dose was approximately 100 to 150 mg per kg of body weight. The dose used for aerosol inhalation was 300 to 500 mg in a 1-ml volume administered three times daily by Vaponefrin nebulizer in two older children.

The clinical condition, hemogram, roentgenological examination of the chest, and cultural studies of the respiratory tract of these patients, including sensitivity tests against penicillin G and DMP, were evaluated prior to and following DMP therapy.

Clinical response was measured by general improvement, fewer coughing spells, decreased rales, lowering of temperature, and reduction of the leukocyte count. The response was considered improved if three or four of these criteria were met, and considered unchanged if only the general condition improved, but other abnormal findings persisted. Because of the marked variation in the natural

course of the respiratory infection of these patients, reduction in coughing spells and improvement in the general condition, without changes in physical findings and leukocyte count, can be considered nonspecific and, therefore, these patients are classified as unchanged in their response to DMP treatment. Roentgenograms of the chest taken pre- and post-treatment were compared with each other.

TABLE 1. *Pertinent data from patients treated with 2,6-dimethoxyphenyl penicillin*

| Patient | Age | Weight | Total daily dose | Duration | Total dose |
|---|---|---|---|---|---|
| | *yr* | *kg* | *g* | *days* | *g* |
| L. C. | 5½ | 17 | 1.7 | 8 | 13.6 |
| G. C. | 8 | 22 | 2.2 | 15 | 33.0 |
| C. C. | 5 | 13 | 1.3 | 28 | 36.4 |
| | | 13 | 1.4 | 12 | 16.8 |
| | | 13 | 1.3 | 20 | 26.0 |
| R. M. | 5 | 17 | 1.5 | 25 | 37.5 |
| D. P. | 8 | 16 | 1.6 | 28 | 44.8 |
| | | 17 | 1.8 | 27 | 48.6 |
| H. R. | 1 | 8 | 0.8 | 45 | 36.0 |
| P. R. | 2¼ | 9 | 1.0 | 23 | 23.0 |
| R. S. | 13½ | 51 | 3.0 | 21 | 63.0 |
| D. T. | 1½ | 10 | 1.0 | 23 | 23.0 |
| M. S. | 3½ | 16 | 1.7 | 14 | 23.8 |
| D. C. | 3 | 10 | 1.5 | 13 | 19.5 |
| S. F. | 4 | 15.5 | 1.6 | 16 | 25.6 |
| J. V. | 6½ | 16 | 2.0 | 18 | 36.0 |
| D. R. | ½ | 4 | 0.4 | 21 | 8.4 |

Serum samples were obtained for DMP assay at varying intervals following the administration of single doses of 25 and 50 mg/kg, intramuscularly. Serum and bronchial secretions were obtained simultaneously for assay at intervals following single doses of DMP administered intramuscularly or by aerosol inhalation.

Susceptibility of *S. aureus* to DMP and penicillin G was determined by the standard tube-dilution method. The source of these strains will be described subsequently. A modification of the penicillin cylinder agar diffusion technique using *Sarcina lutea* as the test organism was adapted for DMP assay.

## Results

According to the criteria stated above, seven of 14 patients showed improvement following eight courses of DMP therapy (Table 2). The two patients who received aerosol therapy concurrently with intramuscular therapy did not respond better than the other patients.

In general, the results of the clinical response correlated well with changes noted in roentgenological examination of the chest. Younger children with more acute inflammatory pulmonary disease responded better than did the older ones with more chronic respiratory involvement.

TABLE 2. *Results of treatment of CF patients with 2,6-dimethoxyphenyl penicillin*

| Patient | Clinical response | Roentgenogram of chest (pre-treatment vs. post-treatment | Respiratory flora (with phage types) | | Side effects |
|---|---|---|---|---|---|
| | | | Pretreatment | Post-treatment | |
| L. C. | Unchanged | Unchanged | *S. aureus* 80/81 | Negative | Fever, skin rash |
| G. C. | Unchanged | Minimal improvement | *S. aureus* 80/81<br>*P. aeruginosa* | *P. aeruginosa*<br>*A. aerogenes* | None |
| C. C. | Slight improvement | Minimal improvement | *S. aureus* 80/81<br>*P. aeruginosa* | *P. aeruginosa* | None |
| | Unchanged | Unchanged | *S. aureus* 80/81<br>*P. aeruginosa* | *P. aeruginosa* | None |
| | Definite improvement | Definite improvement | *S. aureus* 80/81<br>*P. aeruginosa* | *P. aeruginosa* | None |
| R. M. | Slight improvement | Unchanged | *S. aureus* 77/83<br>*P. aeruginosa* | *S. aureus* 7/77<br>*P. aeruginosa* | Hematuria<br>Albuminuria |
| D. P. | Unchanged | Unchanged | *S. aureus* 7<br>*P. aeruginosa* | *P. aeruginosa* | None |
| | Unchanged | Unchanged | *S. aureus* 77 | *P. aeruginosa* | None |
| H. R. | Improvement | Definite improvement | *A. aureus* (NT)*<br>*P. aeruginosa* | Coliform | None |
| P. R. | Unchanged | Unchanged | *S. aureus* 80/81<br>*P. aeruginosa* | *P. aeruginosa* | Fever, leukopenia<br>Neutropenia |
| R. S. | Unchanged | Unchanged | *S. aureus* 7 | Nonsignificant | None |
| D. T. | Definite improvement | Definite improvement | *S. aureus* 53/77/83 | *P. aeruginosa* | Fever, skin rash |
| M. S. | Definite improvement | Not done | *S. aureus* (NT) | *S. aureus* (NT) | None |
| D. C. | Unchanged | Unchanged | *S. aureus* 77<br>*P. aeruginosa* | *S. aureus* 77<br>*P. aeruginosa* | None |
| S. F. | Unchanged | Unchanged | *S. aureus* (NT)<br>*P. aeruginosa* | *S. aureus* 77<br>*P. aeruginosa* | Albuminuria<br>Hematuria |
| J. V. | Slight improvement | Minimal improvement | *S. aureus* 80/81 | *P. aeruginosa*<br>Coliform | None |
| D. R. | Definite improvement | Minimal improvement | *S. aureus* (NT)<br>Coliform | *P. aeruginosa*<br>Coliform | None |

*(NT) = Not typable.

Cultures of respiratory flora, before and immediately after DMP therapy, showed that all patients harbored coagulase-positive *S. aureus*, eight of the 14 patients also harbored *Pseudomonas aeruginosa*, and another also had a coliform organism (Table 2). Cultures performed three days after initiation of therapy and immediately after completion of therapy were negative for coagulase-positive *S. aureus* following 13 of the 17 courses of therapy. However, in all patients, *S. aureus* reappeared in the pharyngeal and nasopharyngeal cultures performed 1 to 6 weeks after discontinuation of therapy.

All strains of *S. aureus*, isolated before and at intervals after discontinuation of DMP therapy, were uniformly inhibited by DMP in concentrations of 0.4 to 3.12 μg/ml.

The results of susceptibility testing of 53 strains of *S. aureus* isolated from these 14 patients before and following DMP therapy and those isolated from 14 other patients with cystic fibrosis are shown in Fig. 1. The sensitivity of these organisms to penicillin G is also included for comparison. Bactericidal level of DMP was determined in 53 strains of *S. aureus*. The results showed that the bactericidal levels and the bacteriostatic levels were the same in 50% of these organisms, and in the remainder of the strains the bactericidal level was two- to fourfold higher than the bacteriostatic level. The sensitivity patterns of strains isolated from DMP-treated and from untreated CF patients were similar.

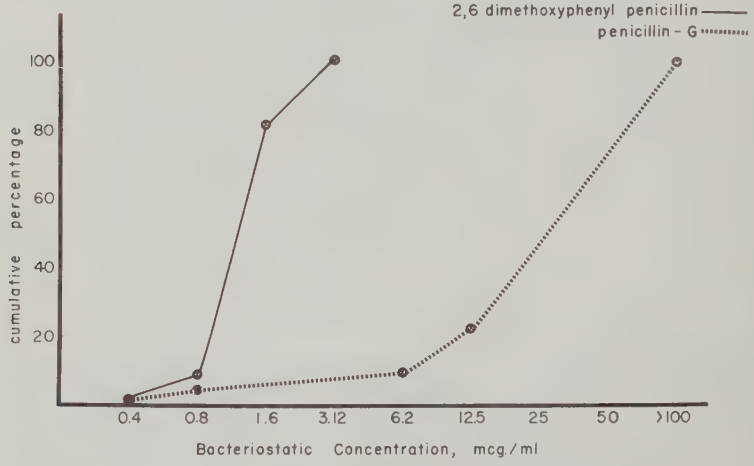


FIG. 1. *Bacteriostatic concentrations of penicillin G and 2,6-dimethoxyphenyl penicillin against 53 strains of Staphylococcus aureus.*

Serum concentrations of DMP were obtained at varying time intervals following the intramuscular administration of single doses of 25 and 50 mg/kg, respectively (Fig. 2). The results revealed a much higher peak concentration of DMP in the serum following a single dose of 50 mg/kg than that detected after a single dose of 25 mg/kg. Oral administration of probenecid, 1 hr prior to an intramuscular dose, maintained higher blood levels, but the serum levels fell rapidly to less than 2 μg/ml at the end of 6 hr. Therefore, if continuous serum levels are desired, the agent should be administered at 6-hr intervals.

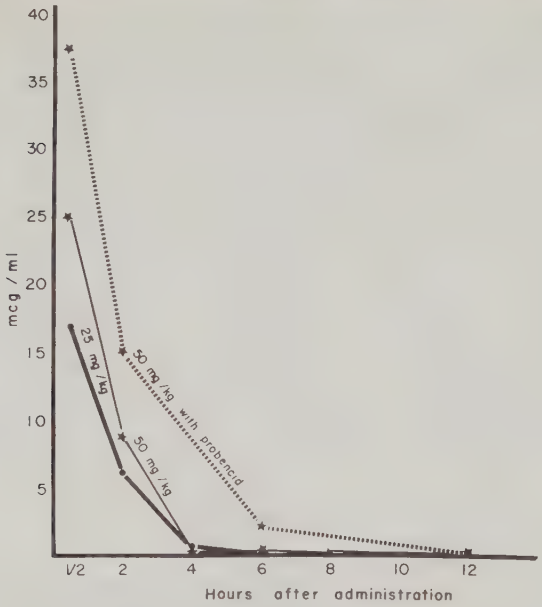


FIG. 2. *Average concentration of 2,6-dimethoxyphenyl penicillin in sera after a single intramuscular dose.*

DMP concentrations in serum and bronchial secretions were determined at varying time intervals after the administration of single doses of DMP by intramuscular and aerosol routes (Fig. 3 and 4). The results indicated some diffusion of DMP from the serum into the bronchial secretion following intramuscular administration. Because of the marked susceptibility of *S. aureus* to DMP, a low level in the bronchial secretions has a suppressive effect on the bacterial flora of the bronchial secretion. It is interesting to note that as long as 12 hr after an intramuscular dose of 50 mg/kg, there was a higher level in the bronchial secretion than in the serum. Figure 4 shows a high level in bronchial secretion following aerosol inhalation but the serum level was low. Therefore, administration of DMP by both intramuscular injection and aerosol inhalation should provide high levels in both serum and bronchial secretions. However, in the two patients in this series who received DMP by both routes, the therapeutic effect was not different from others who were treated with intramuscular injections alone.

*Side effects.* Two patients in this group developed generalized papular erythematous skin rashes of an urticaria nature after 2 and 3 weeks of therapy, respectively. One of these patients developed a

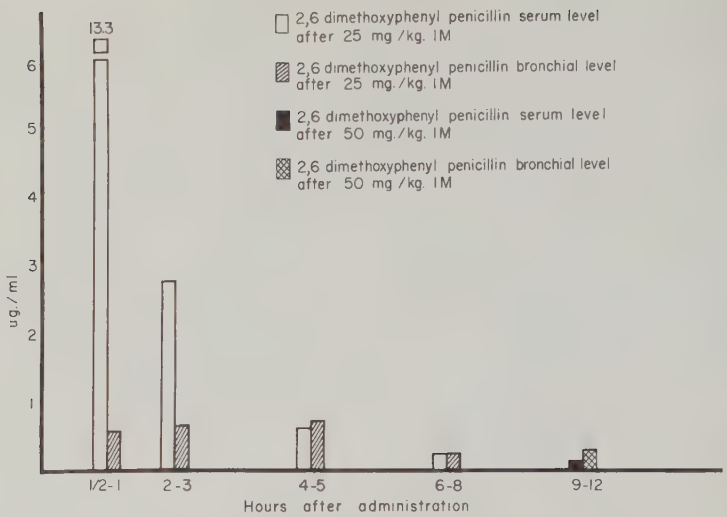


FIG. 3. *2,6-Dimethoxyphenyl penicillin concentration in bronchial secretions and sera after a single intramuscular dose.*

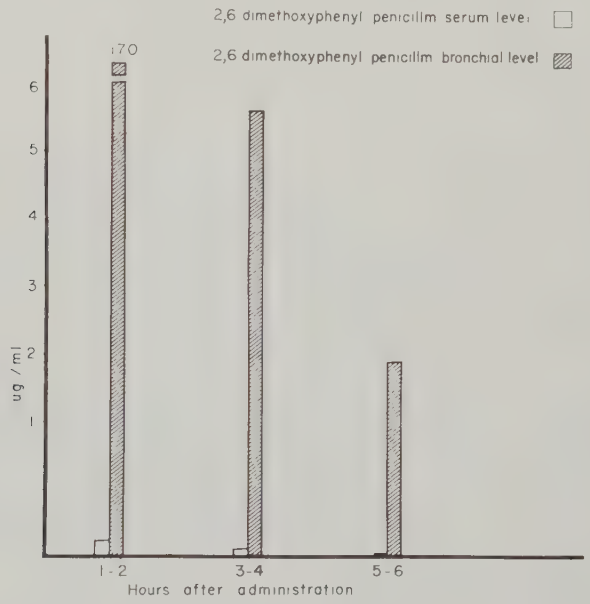


FIG. 4. *2,6-Dimethoxyphenyl penicillin concentration in bronchial secretions and sera after aerosol inhalation of single doses of 300 to 500 mg.*

moderate fever for 3 days before the appearance of the skin rash. Both of the patients had previously received penicillin G and had no allergic manifestations to it. Intradermal tests with various doses of penicillin G and DMP from 10 to 1,000 μg per ml showed no evidence of cutaneous hypersensitivity in either of these patients with skin rash. The serum samples of these patients were tested for circulating antibodies against penicillin G and DMP by the hemagglutination method, but were negative. Another patient had a high continuous fever, associated with leukopenia and neutropenia 17 days after the initiation of therapy (McElfresh and Huang, 1962). Two other patients presented frequency and urgency of urination. Urinalysis showed albuminuria, hematuria, and pyuria, but there was no elevation of blood urea nitrogen. All the untoward effects, including skin rash, fever, leukopenia, and urinary disturbance, subsided promptly when therapy was discontinued. All patients complained of pain at the site of the injection but there was no abscess formation in the patients treated.

## Discussion

The results of this study indicate that DMP is an effective agent for elimination of resistant *S. aureus* responsible for the respiratory infection of CF patients. However, the bacteriological improvement is transient. In the majority of the patients, there was prompt recolonization of *S. aureus* of the same or different phage type in the respiratory tract of these patients. All strains of *S. aureus*, isolated before and after therapy, remained very sensitive to DMP. Thus far, a resistant strain has not been encountered.

The clinical response of cystic fibrosis is more pronounced in younger patients with acute inflammatory process than in older ones with a chronic low-grade infection. This agent inhibits the growth of *S. aureus* in low concentrations easily achievable in serum and bronchial secretion after intramuscular administration. Aerosol inhalation of DMP has been administered without adverse effect. Further study is indicated to determine the effectiveness of intensive therapy with aerosol administration alone.

Intramuscular administration of DMP has resulted in side effects, including fever, skin rashes, neutropenia, leukopenia, albuminuria, hematuria, and pyuria. However, these toxic effects promptly disappeared following discontinuation of therapy in the patients presently reported.

## Acknowledgments

The authors wish to express their gratitude to Edith Cheng and Isabelle Bender for their technical assistance.

## Literature Cited

Huang, N. 1957. Bronchopulmonary suppurative disease, differential diagnosis and management. Ped. Cl. N. Am., Feb. W. B. Saunders Company, Philadelphia.

McElfresh, A. E., and N. N. Huang. 1962. Bone marrow depression resulting from the administration of 2-6 dimethoxyphenyl penicillin with a comment on the value of serum iron determination. New Engl. J. Med. **266**:246-247.

di Sant'agnese, P. A. 1956. Fibrocystic disease of the pancreas, a generalized disease of exocrine glands. J. Am. Med. Assoc. **160**:846-853.

Schwachman, H. 1960. Therapy of cystic fibrosis of pancreas. Pediatrics **25**:155-163.

# IN VITRO ANTIMICROBIAL ACTIVITY OF A SERIES OF SUBSTITUTED 2-IMIDAZOLINES

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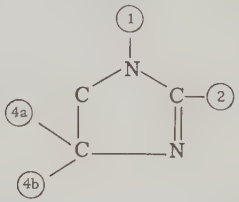
For several years our group has studied the antimicrobial and pharmacological properties of hexahydropyrimidines and the influence of substitutions at the 1,3,5 positions on such activities (Edwards et al., 1956; Church, Read, and Sanders, 1957; Read, Sanders, and Church, 1957; Fredell, White, and Sanders, 1958; Hexahydropyrimidine Symposium, 1958; Barkley et al., 1961). Our experience with these compounds led us to similar studies with 2-imidazolines, the antimicrobial properties of which have been known for several years (Thurston et al., 1946; Wellman and McCallan, 1946; Kyrides et al., 1947). This paper deals with the influence of 1,2,4 substitutions on in vitro antibacterial, antifungal, and antiprotozoal activity. The basic imidazoline moiety and the positions investigated are indicated:




Our preliminary in vitro resistance studies with a representative member of this series will also be reported.

## Materials and Methods

*Compounds.* In the number 1 position, the most frequently occurring substitution was a 2-ethylhexyl group, although other groups such as aminomethyl, methylpropyl, and hydroxyethyl were also employed. A great variety of substitutions was made in the 2 position. The 4a and 4b position contained, most frequently, methyl and

2-ethylhexylaminomethyl groups. The compounds with their substituent groups are listed in Table 1.

TABLE 1. *Series of 1,2,4- substituted imidazolines tested for antimicrobial activity*

| Compound no. | Salt | Position 1 substituents | Position 2 substituents | Position 4a substituents | Position 4b substituents |
|---|---|---|---|---|---|
| W2416 | - | 2-ethylhexyl | 4-methoxybenzyl | methyl | 2-ethylhexylaminomethyl |
| W2460 | - | 2-ethylhexyl | *p*-tolyl | methyl | 2-ethylhexylaminomethyl |
| W2460A | Dioxalate | 2-ethylhexyl | *p*-tolyl | methyl | 2-ethylhexylaminomethyl |
| W3015 | - | 2-ethylhexyl | *m*-tolyl | methyl | 2-ethylhexylaminomethyl |
| W3035 | - | 2-ethylhexyl | 3-phenoxypropyl | methyl | 2-ethylhexylaminomethyl |
| W3089 | - | 2-ethylhexyl | *p*-fluorophenyl | methyl | 2-ethylhexylaminomethyl |
| W3141 | - | 2-ethylhexyl | *p*-hexyloxyphenyl | methyl | 2-ethylhexylaminomethyl |
| W2463 | - | 2-ethylhexyl | benzyl | methyl | 2-ethylhexylaminomethyl |
| W2971 | - | 2-ethylhexyl | 2-furyl | methyl | 2-ethylhexylaminomethyl |
| W3014 | - | 2-ethylhexyl | phenyl | methyl | 2-ethylhexylaminomethyl |
| W3016 | - | 2-ethylhexyl | 3,4-dimethoxyphenyl | methyl | 2-ethylhexylaminomethyl |
| W3017 | - | 2-ethylhexyl | tridecyl | methyl | 2-ethylhexylaminomethyl |
| W3061 | - | 2-ethylhexyl | diphenylmethyl | methyl | 2-ethylhexylaminomethyl |
| W3062 | - | 2-ethylhexyl | *p*-chlorobenzyl | methyl | 2-ethylhexylaminomethyl |
| W3088 | - | 2-ethylhexyl | *p*-methoxyphenyl | methyl | 2-ethylhexylaminomethyl |
| W3140 | - | 2-ethylhexyl | 4-biphenylyl | methyl | 2-ethylhexylaminomethyl |
| W2526 | - | 2-ethylhexyl | methyl | methyl | 2-ethylhexylaminomethyl |
| W2700 | Dihydrochloride | 2-ethylhexyl | amino | methyl | 2-ethylhexylaminomethyl |
| W2970 | - | 2-ethylhexyl | 3,4-dimethoxybenzyl | methyl | 2-ethylhexylaminomethyl |
| W3041 | - | 2-ethylhexyl | phenethyl | methyl | 2-ethylhexylaminomethyl |
| W3068A | Dioxalate | 2-ethylhexyl | *p*-nitrobenzyl | methyl | 2-ethylhexylaminomethyl |
| W2464 | - | 2-ethylhexyl | *p*-chlorophenyl | methyl | 2-ethylhexylaminomethyl |
| W3157 | - | 2-ethylhexyl | *p*-bromobenzyl | methyl | 2-ethylhexylaminomethyl |
| W3158 | - | 2-ethylhexyl | 3,4-dichlorophenyl | methyl | 2-ethylhexylaminomethyl |
| W3087 | - | 2-ethylhexyl | *p*-hydroxyphenyl | methyl | 2-ethylhexylaminomethyl |
| W3286 | - | 2-ethylhexyl | {*p*-{2-[2-(2-methoxyethoxy)ethoxy]ethoxy}phenyl} | methyl | 2-ethylhexylaminomethyl |
| W2684 | - | 2-ethylhexyl | 4-pyridyl | methyl | 2-ethylhexylaminomethyl |
| W2770 | Dihydrobromide | 2-aminoethyl | benzyl | hydrogen | hydrogen |
| W3063 | - | hydrogen | *p*-tolyl | methyl | methyl |
| W2871 | - | 1-methylpropyl | *p*-tolyl | methyl | 1-methylpropylaminomethyl |
| W2680 | Hydrochloride | hydrogen | benzyl | hydrogen | hydrogen |
| W2389 | Hydriodide | hydrogen | 2-phenylhydrazino | hydrogen | hydrogen |
| W2711 | Hydriodide | hydrogen | 2-benzylidene-hydrazino | hydrogen | hydrogen |
| W3170 | Hydriodide | hydrogen | hydrazino | hydrogen | hydrogen |
| W2969 | - | 2-hydroxyethyl | *p*-tolyl | hydrogen | hydrogen |
| W2758 | Dihydrobromide | 2-aminoethyl | methyl | hydrogen | hydrogen |
| W2740 | Dihydrobromide | 2-aminoethyl | *p*-tolyl | hydrogen | hydrogen |
| W2835 | - | 2-dimethylamino-ethyl | *p*-tolyl | hydrogen | hydrogen |

*Antimicrobial activity.* The test microorganisms were obtained from either the American Type Culture Collection, our own stock culture collection, or recent isolates from patients. The latter two types are designated by the letter "M" and those strains that are resistant to one or more antibiotics are designated by the letter "R", following the strain number. The test organisms employed were as follows: *Staphylococcus aureus* 6538P, *Streptococcus faecalis* M40, *Bacillus subtilis* M79, *Bacillus megaterium* M62, *Pseudomonas aeruginosa* 9027, *Proteus vulgaris* 881, *Escherichia coli* 6883, *E. coli* M159R, *Aerobacter aerogenes* M55, *Candida albicans* 10231, *Cryptococcus neoformans* M309F, *Trichophyton mentagrophytes* M320F, *Epidermophyton floccosum* 10227, *Sporotrichum schenckii* M319F, *Asperigillus*

*niger* M302F, *Endamoeba histolytica* M1P (Schaudinn-F22T), and *Trichomonas vaginalis* M2P (Donne-Strain #1).

The antimicrobial activity of the imidazolines was determined by the usual method of twofold or threefold serial dilutions. The following media were used: for bacteria, tryptose phosphate broth, with and without 20% horse serum; for fungi, Sabouraud dextrose broth, with and without 20% horse serum; for *Endamoeba histolytica,* coagulated egg slants, overlaid with Locke's buffered saline with sterile rice powder; and for *Trichomonas vaginalis,* trichosel broth (Baltimore Biological Laboratory, Inc.). For bacteria, *Cryptococcus,* and *Candida,* 0.1 ml of a 1:1000 dilution of an 18-hr culture was used as the inoculum. For the filamentous fungi, the inoculum was a 3-mm agar plug from a 10 to 15 day culture maintained on Sabouraud dextrose agar. For *E. histolytica* 0.1 ml and for *T. vaginalis* 0.15 ml of a 48-hr culture was used.

The bacterial and protozoal tests were held for 48 hr at 37 C and the fungal tests for 72 hr at 25 C. The minimal inhibitory concentration (MIC) was recorded for each organism as the lowest concentration of the compound at which there was no visible growth. Staining and subculturing were used when it was necessary to substantiate the end points. The antiprotozoal tests were evaluated microscopically. The MIC was recorded as the lowest concentration of the compound at which no motile cells could be seen in at least 20 fields.

*In vitro resistance study.* By use of compound W2460, a group of sensitive microorganisms was transferred serially in subinhibitory concentrations to determine resistance development. This was accomplished by inoculating a series of twofold dilutions of the compound in appropriate growth medium (tryptose phosphate broth for the bacteria, and Sabouraud dextrose broth for *C. albicans)* with 0.1 ml of a 1:1000 dilution of an 18-hr broth culture. After incubation, the inoculum for the next passage was taken from the tube containing $\frac{1}{4}$ of the MIC. A total of 15 passages was made.

## Results and Discussion

In the series of compounds studied, only those containing a 2-ethylhexyl group in the 1-position and a methyl and a 2-ethylhexylaminomethyl group in the 4-position were active against the test organisms. The effect on activity of various substituents at position 2 in compounds containing the 1,4 moieties was varied and depended on the test organisms.

The antibacterial activities of the active compounds were consistently good against the gram-positive bacteria, regardless of the 2-substitution. All of these compounds were uniformly poor in activity against *Pseudomonas* and *Proteus.* Against *Escherichia* and

*Aerobacter*, however, large differences in activity were exhibited, depending on the 2-position substitution. These data may be seen in Table 2. The reduction of activity in the presence of serum showed

TABLE 2. *In vitro antibacterial activity of substituted imidazolines*

| Compound no. | Minimal inhibitory concentration in μg/ml | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | *S. aureus* 6538P | *S. faecalis* M40 | *B. subtilis* M79 | *B. megaterium* M62 | *P. aeruginosa* 9027 | *P. vulgaris* 881 | *E. coli* 6883 | *E. coli* M159R | *A. aerogenes* M55 |
| W2416 | <4 | <4 | <4 | <4 | 375 | 1,500 | 11 | 1,500 | 11 |
| W2416 + 20% H.S.* | 62 | 62 | 62 | 125 | 750 | >1,500 | 188 | >1,500 | 188 |
| W2460 | <4 | <4 | <4 | <4 | 750 | >1,500 | <4 | <4 | 4 |
| W2460 + 20% H.S. | 62 | 62 | 62 | 62 | >1,500 | >1,500 | 188 | 188 | 188 |
| W2460A | <4 | <4 | <4 | <4 | >1,000 | >1,000 | 31 | 62 | 31 |
| W2460A + 20% H.S. | 62 | 62 | 62 | 125 | >1,000 | >1,000 | 500 | 500 | 62 |
| W3015 | <4 | <4 | <4 | <4 | 1,000 | 1,000 | 31 | 31 | >1,000 |
| W3015 + 20% H.S. | 62 | 62 | 125 | 250 | >1,000 | >1,000 | >1,000 | >1,000 | >1,000 |
| W3035 | <4 | <4 | <4 | <4 | >1,000 | >1,000 | 16 | 16 | 31 |
| W3035 + 20% H.S. | 62 | 62 | 31 | 62 | >1,000 | >1,000 | >1,000 | >1,000 | >1,000 |
| W3089 | <4 | <4 | <4 | <4 | >1,000 | >1,000 | 500 | 31 | >1,000 |
| W3089 + 20% H.S. | 62 | 62 | 62 | 62 | >1,000 | >1,000 | >1,000 | >1,000 | >1,000 |
| W3141 | <4 | <4 | <4 | <4 | 1,000 | 1,000 | 500 | 250 | 250 |
| W3141 + 20% H.S. | 31 | 31 | 62 | 125 | 1,000 | >1,000 | 1,000 | 1,000 | 500 |
| W2463 | <4 | <4 | <4 | <4 | 750 | 1,500 | 11 | <4 | 23 |
| W2463 + 20% H.S. | 62 | 62 | 31 | 62 | 1,500 | >1,500 | 375 | 375 | 375 |
| W2971 | <4 | <4 | <4 | <4 | >1,000 | >1,000 | 16 | <4 | 125 |
| W2971 + 20% H.S. | 62 | 62 | 62 | 62 | >1,000 | >1,000 | 1,000 | 62 | >1,000 |
| W3014 | <4 | <4 | <4 | <4 | >1,000 | >1,000 | 16 | 31 | 1,000 |
| W3014 + 20% H.S. | 62 | 62 | 62 | 62 | >1,000 | >1,000 | >1,000 | >1,000 | >1,000 |
| W3016 | <4 | <4 | <4 | <4 | 1,000 | >1,000 | 62 | 62 | 125 |
| W3016 + 20% H.S. | 62 | 62 | 62 | 62 | >1,000 | >1,000 | 500 | 1,000 | 500 |
| W3017 | 16 | 8 | 16 | 16 | >1,000 | >1,000 | >1,000 | >1,000 | >1,000 |
| W3017 + 20% H.S. | 1,000 | 1,000 | 250 | 250 | >1,000 | >1,000 | >1,000 | >1,000 | >1,000 |
| W3061 | <4 | <4 | <4 | <4 | 1,000 | 1,000 | 1,000 | 1,000 | 1,000 |
| W3061 + 20% H.S. | 125 | 125 | 62 | 62 | >1,000 | >1,000 | >1,000 | >1,000 | >1,000 |
| W3062 | <4 | <4 | <4 | <4 | >1,000 | >1,000 | 16 | 16 | 31 |
| W3062 + 20% H.S. | 62 | 62 | 62 | 125 | >1,000 | >1,000 | 1,000 | 1,000 | 1,000 |
| W3088 | <4 | <4 | <4 | <4 | 500 | >1,000 | 31 | 31 | <4 |
| W3088 + 20% H.S. | 62 | 125 | 31 | 62 | 1,000 | >1,000 | 1,000 | 1,000 | 250 |
| W3140 | <4 | <4 | <4 | <4 | 1,000 | 1,000 | 1,000 | 1,000 | 250 |
| W3140 + 20% H.S. | 250 | 125 | 125 | 125 | >1,000 | >1,000 | >1,000 | >1,000 | 500 |
| W2526 | 8 | 8 | 31 | 16 | 375 | 750 | <4 | <4 | 23 |
| W2526 + 20% H.S. | 62 | 62 | 62 | 62 | >1,500 | >1,500 | 23 | 188 | 47 |
| W2700 | <4 | <4 | 8 | 8 | 62 | 1,000 | <4 | 8 | 16 |
| W2700 + 20% H.S. | >1,000 | >1,000 | 250 | 250 | 500 | >1,000 | 125 | 250 | >1,000 |
| W2970 | <4 | <4 | <4 | <4 | >1,000 | >1,000 | 16 | 62 | 125 |
| W2970 + 20% H.S. | 62 | 62 | 62 | 62 | >1,000 | >1,000 | >1,000 | 500 | 1,000 |
| W3041 | <4 | <4 | <4 | <4 | 500 | >1,000 | 31 | 8 | <4 |
| W3041 + 20% H.S. | 125 | 62 | 31 | 31 | >1,000 | >1,000 | >1,000 | >1,000 | >1,000 |
| W3068A | <4 | <4 | <4 | <4 | >1,000 | >1,000 | 31 | 31 | 31 |
| W3068A + 20% H.S. | 62 | 62 | 250 | 125 | >1,000 | >1,000 | >1,000 | >1,000 | >1,000 |
| W2464 | <4 | <4 | <4 | <4 | 375 | >1,500 | >1,500 | >1,500 | >1,500 |
| W2464 + 20% H.S. | 250 | 250 | 125 | 250 | 375 | >1,500 | >1,500 | >1,500 | >1,500 |
| W3157 | <4 | <4 | <4 | <4 | >1,000 | >1,000 | >1,000 | >1,000 | >1,000 |
| W3157 + 20% H.S. | 125 | 125 | 125 | 125 | >1,000 | >1,000 | >1,000 | >1,000 | >1,000 |
| W3158 | <4 | <4 | <4 | <4 | >1,000 | >1,000 | >1,000 | >1,000 | >1,000 |
| W3158 + 20% H.S. | 500 | 500 | 500 | 500 | >1,000 | >1,000 | >1,000 | >1,000 | >1,000 |
| W3087 | <4 | <4 | <4 | <4 | >1,000 | >1,000 | 16 | 16 | 62 |
| W3087 + 20% H.S. | 125 | 125 | 62 | 62 | >1,000 | >1,000 | >1,000 | >1,000 | 500 |
| W3286 | <4 | <4 | <4 | <4 | 500 | 1,000 | 500 | 250 | 500 |
| W3286 + 20% H.S. | 62 | 125 | 31 | 62 | 1,000 | 1,000 | 1,000 | 1,000 | >1,000 |
| W2684 | <4 | <4 | <4 | <4 | - | - | - | - | - |
| W2684 + 20% H.S. | 62 | 62 | 62 | 62 | - | - | - | - | - |
| W2770 | | | | | | | | | |
| W3063 | | | | | | | | | |
| W2871 | | | | | | | | | |
| W2680 | | | | | | | | | |
| W2389 | | | | | | | | | |
| W2711 | Inactive against all test bacteria at 1,000 μg/ml. | | | | | | | | |
| W3170 | | | | | | | | | |
| W2969 | | | | | | | | | |
| W2758 | | | | | | | | | |
| W2740 | | | | | | | | | |
| W2835 | | | | | | | | | |

*H.S. = Horse serum.

considerable variation among the compounds. The degree of serum inactivation is not, in general, correlated with activity in the absence of serum (Table 2).

Many of the compounds exhibited considerable activity against the fungi. The activity of a particular compound on a particular species was dependent on the substituent at position 2 (Table 3). The same was true for its behavior in serum.

TABLE 3. *In vitro antifungal activity of substituted imidazolines*

| Compound no. | Minimal inhibitory concentration in μg/ml | | | | | |
|---|---|---|---|---|---|---|
| | *C. albicans* 10231 | *C. neoformans* M309F | *T. mentagrophytes* M320F | *E. floccosum* 10227 | *S. schenckii* M319F | *A. niger* M302F |
| W2416 | 23 | 16 | 6 | 16 | 23 | 125 |
| W2416 + 20% H.S.* | 94 | 125 | 23 | 22 | 23 | 250 |
| W2460 | 47 | 5 | 11 | <4 | 11 | 250 |
| W2460 + 20% H.S. | 94 | 23 | 47 | 16 | 23 | 500 |
| W2460A | 125 | 31 | 8 | 31 | 62 | 125 |
| W2460A + 20% H.S. | 1,000 | 250 | 62 | 125 | 250 | 500 |
| W3015 | 62 | 250 | 16 | 62 | 250 | 250 |
| W3015 + 20% H.S. | 250 | 250 | 62 | 62 | 250 | 250 |
| W3035 | 16 | 8 | 8 | 8 | 16 | 125 |
| W3035 + 20% H.S. | 250 | 125 | 125 | 125 | 125 | 250 |
| W3089 | 125 | 8 | 8 | 8 | 62 | 250 |
| W3089 + 20% H.S. | 250 | 250 | 31 | 250 | 62 | 250 |
| W3141 | 8 | 8 | 31 | 125 | 16 | 500 |
| W3141 + 20% H.S. | 500 | 62 | 125 | 500 | 62 | 1,000 |
| W2463 | 47 | 62 | 23 | 8 | 125 | 250 |
| W2463 + 20% H.S. | 375 | 125 | 94 | 31 | 500 | 500 |
| W2971 | 250 | 250 | 62 | 62 | 125 | 500 |
| W2971 + 20% H.S. | 1,000 | 1,000 | 125 | 250 | 1,000 | 500 |
| W3014 | 250 | 16 | 8 | 31 | 62 | 250 |
| W3014 + 20% H.S. | >1,000 | 125 | 62 | 62 | 250 | 500 |
| W3016 | 125 | 31 | 8 | 31 | 62 | 250 |
| W3016 + 20% H.S. | 1,000 | 125 | 125 | 125 | 125 | 500 |
| W3017 | <4 | 16 | 16 | 31 | 62 | 62 |
| W3017 + 20% H.S. | 125 | 125 | 250 | 125 | 250 | 250 |
| W3061 | <4 | <4 | 31 | 8 | 16 | 250 |
| W3061 + 20% H.S. | 250 | 250 | 125 | 250 | 1,000 | 250 |
| W3062 | 125 | 62 | <4 | 62 | 31 | 250 |
| W3062 + 20% H.S. | 1,000 | 125 | 125 | 125 | 125 | 500 |
| W3088 | 12 | <4 | <4 | 125 | <4 | 500 |
| W3088 + 20% H.S. | 125 | 31 | <4 | 500 | 62 | 1,000 |
| W3140 | 8 | 8 | 62 | 8 | <4 | 125 |
| W3140 + 20% H.S. | 250 | 31 | 125 | 250 | 500 | 250 |
| W2526 | 188 | 94 | 94 | 62 | 188 | 500 |
| W2526 + 20% H.S. | 750 | 750 | 333 | 250 | 188 | 1,000 |
| W2700 | - | 125 | - | 16 | 500 | 500 |
| W2700 + 20% H.S. | - | 500 | - | 125 | 1,000 | 1,000 |
| W2970 | 16 | 250 | 62 | 62 | 125 | 500 |
| W2970 + 20% H.S. | 1,000 | 250 | 250 | 125 | 125 | 500 |
| W3041 | 62 | <4 | 8 | 125 | 16 | 500 |
| W3041 + 20% H.S. | >1,000 | 125 | 125 | 250 | 1,000 | >1,000 |
| W2146 | 37 | - | 12 | 111 | - | 31 |
| W2146 + 20% H.S. | 1,000 | - | 333 | 250 | - | >1,000 |
| W3068A | 125 | 31 | 16 | 31 | 62 | 500 |
| W3068A + 20% H.S. | 1,000 | 125 | 250 | - | 1,000 | 500 |
| W2464 | 375 | 125 | 11 | 16 | 125 | 500 |
| W2464 + 20% H.S. | 750 | 188 | 188 | 62 | 188 | 500 |
| W3157 | 62 | <4 | 16 | 8 | 31 | 250 |
| W3157 + 20% H.S. | >1,000 | 1,000 | 62 | 62 | 250 | 500 |
| W3158 | 62 | <4 | 16 | 16 | 31 | 1,000 |
| W3158 + 20% H.S. | >1,000 | >1,000 | 62 | 250 | >1,000 | >1,000 |
| W3087 | 125 | 125 | 250 | 62 | 125 | 250 |
| W3087 + 20% H.S. | >1,000 | 500 | 500 | 250 | >1,000 | 1,000 |
| W3286 | 500 | <4 | 62 | 250 | 16 | 250 |
| W3286 + 20% H.S. | 1,000 | 500 | 125 | 500 | 125 | 1,000 |
| W2684 | 62 | 62 | 37 | - | - | - |
| W2684 + 20% H.S. | 250 | 250 | 125 | - | - | - |
| W2770 | | | | | | |
| W3063 | | | | | | |
| W2871 | | | | | | |
| W2680 | | | | | | |
| W2389 | | | | | | |
| W2711 | Inactive against all test fungi at 1,000 μg/ml. | | | | | |
| W3170A | | | | | | |
| W2969 | | | | | | |
| W2758 | | | | | | |
| W2740 | | | | | | |
| W2835 | | | | | | |

*H.S. = Horse serum.

Many of the imidazolines demonstrated good activity against *E. histolytica* and *T. vaginalis*. The antiprotozoal activity appears also to be dependent on substitutions in the 2-position, as indicated in Table 4.

TABLE 4. *Antiprotozoal activity of a series of substituted imidazolines*

| Compound no. | Minimal inhibitory concentrations in μg/ml | |
|---|---|---|
| | *E. histolytica* (M1P) | *T. vaginalis* (M2P) |
| W2416 | 7.8-15.6 | 1.9- 3.9 |
| W2460 | 15.6-31.2 | 0.48-0.97 |
| W2460A | 31.2-62.5 | 0.48-0.97 |
| W3015 | 7.8-15.6 | 0.48-0.97 |
| W3035 | 15.6-31.2 | 0.9 -1.95 |
| W3089 | 7.8-15.6 | 0.48-0.97 |
| W3141 | 31.2-62.5 | 0.12-0.24 |
| W2463 | 31.2-62.5 | 0.24-0.48 |
| W2971 | 15.6-31.2 | 0.48-0.97 |
| W3014 | 15.6-31.2 | 0.48-0.97 |
| W3016 | 31.2-62.5 | 0.48-0.97 |
| W3017 | 62.5-125 | 0.24-0.48 |
| W3061 | 31.2-62.5 | 0.48-0.97 |
| W3062 | 15.6-31.2 | 0.48-0.97 |
| W3088 | 15.6-31.2 | 0.48-0.97 |
| W3140 | 62.5-125 | 0.12-0.24 |
| W2526 | 62.5-125 | 3.9 -7.8 |
| W2700 | 15.6-31.2 | 3.9 -7.8 |
| W2970 | 31.2-62.5 | 0.9 -1.95 |
| W3041 | 7.8-15.6 | 0.24-0.48 |
| W2068A | 15.6-31.2 | 0.48-0.97 |
| W2464 | 7.8-15.6 | 0.24-0.48 |
| W3087 | 15.6-31.2 | 0.97-1.95 |
| W3063 | 1,000 | 1,000 |
| W2871 | 250-500 | 125-250 |
| W2680 | >1,000 | >1,000 |
| W2711 | 1,000 | 250-500 |
| W2969 | >1,000 | 1,000 |
| W2758 | >1,000 | 1,000 |
| W2740 | >1,000 | >1,000 |
| W2835 | 250-500 | 250-500 |

Compound W2460 [1-(2-ethylhexyl)-4-(2-ethylhexylaminomethyl)-4-methyl-2-*p*-tolyl-2-imidazoline] was noted to have rather good activity against most of the test organisms. Therefore, a study was conducted in order to determine the possibility of resistance development among a group of microorganisms. These results may be seen in Table 5. With the organisms tested, after 15 passages, no resistance developed in the gram-positive organisms or *Candida*, whereas a 15- to 30-fold increase in resistance developed with the gram-

negative bacteria. The resulting cultures consisted of cells considerably elongated and filamentous, reminiscent of hexachlorophene-resistant *E. coli* cells, described previously by Moyer and Wyss (1960). This resistance was stable on three successive subcultures in imadazoline-free media.

TABLE 5. *In vitro resistance studies with W2460*

| Organism | Original MIC | MIC after 15 passages | Factor of increased resistance |
|---|---|---|---|
| | *μg/ml* | *μg/ml* | |
| *Staphylococcus aureus* 6538P | 2 | 2 | 1 |
| *Bacillus subtilis* M76 | 1 | 1 | 1 |
| *Escherichia coli* M52 | 16 | 500 | 31 |
| *Aerobacter aerogenes* M55 | 31 | 500 | 16 |
| *Candida albicans* M306F | 31 | 31 | 1 |

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## Literature Cited

Barkley, F. A., F. J. Turner, R. S. Pianotti, P. L. Carthage, and B. S. Schwartz. 1961. In vitro antibacterial and antifungal activity of some 1,3,5-substituted hexahydropyrimidines. Antimicrobial Agents Annual 1960, p. 507-519.

Church, B. D., R. R. Read, and R. G. Sanders. 1957. Hexahydropyrimidines. Mechanism of action on several microbiological systems. Abstr. Papers Am. Chem. Soc., p. 2N.

Edwards, R. B., B. D. Church, C. D. Dukes, and R. G. Sanders. 1956. The *in vitro* and *in vivo* antibacterial activity of certain hexahydropyrimidines. Bacteriol. Proc., p. 66.

Fredell, W. G., R. C. White, and R. G. Sanders. 1958. Topical applications of a new antibacterial agent. J. Am. Pharm. Assoc., Pract. Pharm. Ed. 19:428-431.

Hexahydropyrimidine Symposium 1958. Northwestern University, Chicago.

Kyrides, L. P., F. B. Zienty, G. W. Steahly, and H. L. Morrill. 1947. Substituted imidazolines and 2-imidazolines. J. Org. Chem. 12:577-596.

Moyer, J. E., and O. Wyss. 1960. Isolation and characterization of a filamentous mutant of *Escherichia coli* B/r resistant to hexachlorophene. Paper 61-22, School of Aviation Medicine, Brooks Air Force Base, Texas.

Reed, R. R., R. G. Sanders, and B. D. Church. 1957. Studies on hexahydropyrimidines. Chemical structure and bacteriological activity. Abstr. Papers Am. Chem. Soc., p. 1-2 N.

Thurston, H. W., Jr., J. B. Harry, F. H. Lewis, A. B. Groves, and C. F. Taylor. 1946. Glyoxalidine derivatives as foliage fungicides. II. Field studies. Contrib. Boyce Thompson Inst. 14: 161-171.

Wellman, R. H., and S. E. A. McCallan. 1946. Glyoxalidine derivatives as foliage fungicides. I. Laboratory studies. Contrib. Boyce Thompson Inst. 14:151-160.

# IN VITRO STUDIES OF SELECTED ANTIMICROBIAL BIGUANIDES

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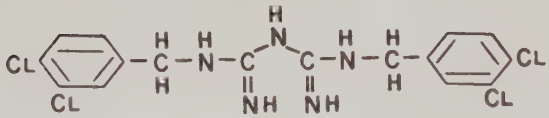
An examination of 242 $N^1$,$N^5$-substituted biguanides revealed that 50% of the compounds were able to prevent the growth in vitro of one or more test microorganisms at concentrations below 10 $\mu$g per ml (Weinberg, 1961). The sensitive cells were killed rapidly by the active compounds, and it was not possible to obtain biguanide-resistant variants. The antibacterial action of the compounds was not affected by the presence, in the medium, of 1.0% aqueous autoclaved extracts of yeast, soybean, liver, brain, or heart. In contrast, a 10- to 100-fold supression of the bacteriostatic activity of the biguanides was obtained in the presence of either 1.0% egg yolk or 1.0% suspensions of homogenized autoclaved particles of yeast, soybean, liver, or brain (but not heart). In the present study, an attempt was made to identify the biguanide-reversing substances and to neutralize their action.

## Materials and Methods

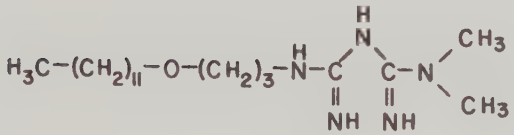
*Compounds, cultures, and cultural conditions.* Two of the most active compounds were selected for intensive investigation: $N^1$,$N^5$-di-(3,4 dichlorobenzyl) biguanide HCl (AM-1) and $N^1$-$\gamma$-lauroxypropyl, $N^5$,$N^5$-dimethyl biguanide HCl (AM-6) (Fig. 1). AM-1 is highly active against gram-positive and gram-negative bacteria whereas AM-6, at low concentrations, inhibits only gram-positive organisms (Table 1). Stock solutions of the hydrochloride salts (1.0% in 95% ethanol) were diluted with deionized water to give working solutions of 500 $\mu$g per

TABLE 1. *Minimal inhibitory concentrations of selected biguanides*

| Test organism | AM-1 | AM-6 |
|---|---|---|
| | *µg per ml* | *µg per ml* |
| *Staphylococcus aureus* | 0.3-1.0 | 1.0-3.0 |
| *Escherichia coli* | 1.0-3.0 | >30 |
| *Candida albicans* | 3.0-10.0 | >30 |



AM-I



AM-6

FIG. 1. *Structural formulas of the active compounds.*

ml. The working solutions were sterilized by autoclaving and then stored at 5 C. Additional concentrations of working solutions were prepared as needed by making further dilutions in sterile deionized water.

The test cultures consisted of stock laboratory strains of coagulase-positive *Staphylococcus aureus*, *Escherichia coli*, and *Candida albicans*. The bacterial strains were grown on nutrient agar slants and the yeast strain on glucose yeast infusion agar slants. Inocula were prepared by diluting a sample of growth in nutrient broth so that the broth contained approximately $1 \times 10^6$ viable cells per ml. The experimental plates consisted of nutrient agar (pH 7.0) or glucose yeast infusion agar (pH 6.0) for the bacterial and yeast strains, respectively. Control plates containing added ethanol were included to make certain that the small quantity of solvent carried over in the diluted solutions of biguanides was not inhibitory. Each agar surface in the experimental and control plates was spread with 0.05 ml of the inoculum and then incubated for 48 hr at 37 C.

*Test substances.* Two groups of substances were tested for possible biguanide-suppressing activity. The first group consisted of concentrations of the nine types of lecithin listed in Fig. 2. The first three types were kindly supplied by Erich Baer (University of Toronto), the fourth and fifth were purchased from California Corporation for Biochemical Research, the sixth from Sigma Chemical Company, and the last three from Nutritional Biochemical Corporation (NBC). The second group consisted of 0.1 and 1.0% concentrations of such nonphosphatide lipid derivatives as cholesterol (Difco), choline (NBC), glycerol (Mallinkrodt), lanolin (Fisher), myristic acid (Matheson), and stearic acid (Fisher).

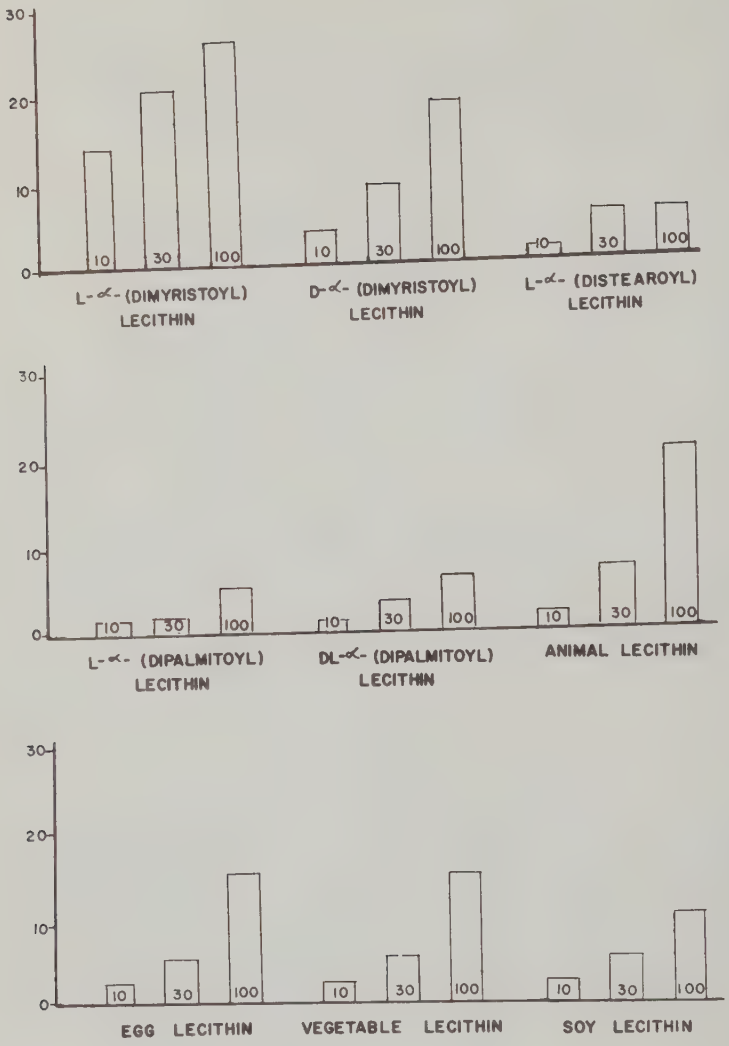



FIG. 2. *Ability of various types of lecithin to suppress the antimicrobial action of the biguanides. The numbers within the bars indicate micrograms of added lecithin per ml of culture medium. The numbers on the ordinate scale indicate the average factor of reversal calculated from data obtained with each of the two biguanides tested against each of the three test microorganisms. The factor of reversal is the ratio of the concentration of biguanide required for inhibition of growth in the presence of lecithin to the concentration needed in the absence of lecithin.*

The following salts were tested for possible neutralization of the biguanide-suppressing action of animal lecithin: the chlorides of aluminum, antimony, bismuth, cadmium, calcium, cobalt, copper, iron, magnesium, nickel, and zinc; and the sodium salts of chloride, ethylenediaminetetraacetate, metavanadate, and molybdate. In each case, the maximum concentration tolerated by the bacteria, as well as 0.1 and 0.01% of this amount, was tested. Also investigated for possible neutralization of the lecithin action were various concentrations of cetyl trimethylammonium bromide (CTAB) (Eastman Organic), Tween 80 (Atlas Powder), and a suspension of homogenized fresh defatted beef heart particles. Each of the test suspensions or solutions of phosphatides, lipid derivatives, salts, CTAB, and heart tissue was sterilized by autoclaving and added aseptically to the medium, along with the biguanides, before the agar was poured into petri plates.

## Results and Discussion

Inasmuch as the particulate suspensions, but not the aqueous extracts of plant, animal, and microbial tissues, contained the biguanide-reversing substance, it was predicted that the active material might be lipid in nature. Of the lipid materials tested, only the nine samples of lecithin were active (Fig. 2). The action of the various lecithins was usually, but not always, slightly greater with AM-1 than with AM-6; and, with AM-1, was approximately the same with either *S. aureus* or *E. coli*. It should be noted, however, that even in the presence of added lecithin, the biguanides possessed a considerable amount of antimicrobial activity. For example, in the presence of as much as 100 $\mu$g per ml of added animal lecithin, the concentration of AM-1 required to kill $1 \times 10^6$ cells of *S. aureus* in nutrient broth in 30 min was between 10 and 30 $\mu$g per ml.

In general, the activity of the samples of lecithin corresponded with the activity of the whole tissue particles based on their phosphatide content. For example, the average biguanide-reversing action of 30 $\mu$g per ml of egg lecithin was fivefold and the activity of 300 $\mu$g per ml of fresh egg yolk (which has a phosphatide content of approximately 10%) was sixfold. Two exceptions were liver and heart tissue; liver tissue was somewhat more active in reversing the biguanides than could be accounted for by its phosphatide content whereas heart tissue was completely inactive.

Inasmuch as the phosphatides suppress the antibacterial action of such surface-active agents as CTAB (Baker, Harrison, and Miller, 1941), several comparative studies of the biguanides and CTAB were performed. The two types of compounds were equally inhibitory towards *S. aureus* and were suppressed to the same extent by animal lecithin. However, CTAB was considerably less active against

*E. coli* and *C. albicans* than was AM-1; indeed, in this respect, CTAB was as inactive as was AM-6.

Combinations of AM-1 and CTAB were neither antagonistic nor synergistic in their antistaphylococcal action, nor could the two compounds protect each other from lecithin suppression. Likewise, neither Tween 80 nor the suspension of heart tissue was able to protect AM-1 from the action of lecithin.

Inasmuch as the phosphatides are precipitated by cadmium ions (Wittcoff, 1951), digested by calcium-activated enzymes (Bloch, 1960), and oxidized by a vanadium-activated enzyme (Bernheim and Bernheim, 1939), it was postulated that appropriate changes in the metal content of the medium might suppress the action of lecithin. However, neither the antistaphylococcal action of AM-1 nor its suppression by lecithin was affected by any of the metal salts or metal binding agents tested. Apparently the maximum nontoxic concentrations of the various metallic ions were neither sufficiently high to precipitate the lecithin nor to activate postulated bacterial lecithinases.

## Acknowledgments

This investigation was supported in part by grant E-4184 from the National Institute of Allergy and Infectious Diseases, U.S. Public Health Service. The excellent technical assistance of Gretchen A. Andis, Ann L. Bockstahler, Judith I. Brooks, Melinda A. Hendry, and Marilyn J. Jones is gratefully acknowledged. The biguanides were synthesized in the laboratories of the U.S. Vitamin and Pharmaceutical Corporation and supplied to the author through the courtesy of S. L. Shapiro.

## Literature Cited

Baker, Z., R. W. Harrison, and B. F. Miller. 1941. The bactericidal action of synthetic detergents. J. Exptl. Med. **74**:611-620.

Bernheim, F., and M. L. C. Bernheim. 1939. The action of vanadium on the oxidation of phospholipids by certain tissues. J. Biol. Chem. **127**:353-360.

Bloch, K. E. 1960. Lipide metabolism. John Wiley and Sons, Inc., New York. p. 188, 209.

Weinberg, E. D. 1961. The antimicrobial activity of $N^1$,$N^5$-substituted biguanides. Antibiotics & Chemotherapy **11**:572-582.

Wittcoff, H. 1951. The phosphatides. Reinhold Publishing Corp., New York. p. 94-96.

# ANTIBACTERIAL AND ANTIFUNGAL SPECTRUM OF 1,2-PROPANEDIOL DIACETATE

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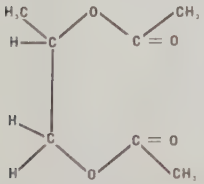
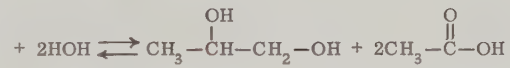
Lower aliphatic acids and alcohols are known to have antibacterial and antifungal action. It was speculated that a molecule which chemically combined these two agents might have superior antimicrobial properties.

The present study concerns the antimicrobial spectrum of 1,2-propanediol diacetate. It is a stable liquid substance in pure form, but will gradually break down into its parent compounds in the presence of moisture.





To maintain stability in a solvent system, acetic acid, which also provides the desirable pH for topical application in clinical practice, is incorporated. The best and most useful composition in such an antimicrobial system has the following composition and is known as VoSol: 1,2-propanediol diacetate, 3%; acetic acid, 2%; and propylene glycol, 95%.

## Materials and Methods

*Preparation of 1,2-propanediol diacetate.* Slowly, 97 g of acetic anhydride are added with constant stirring to a solution of 1 g *p*-toluenesulfonic acid and 34 g of propylene glycol at 80 to 90 C. The reaction mixture is then refluxed for 30 min. Distillation through a fractionating column yields pure 1,2-propanediol diacetate with a boiling point of 189 to 190 C.

*Antibacterial spectrum.* 1,2-propanediol diacetate, VoSol, polymyxin, sulfadiazine, and nitrofurantoin were serially diluted in 10 sterile Kahn tubes containing 0.5 ml brain heart infusion broth (Difco). After the addition of 0.5 ml of $10^{-3}$ dilution of overnight (16 to 18 hr)

cultures of bacteria, the final concentrations of VoSol ranged from 25 to 0.045%; 1,2-propanediol diacetate from 15 to 0.027 mg/ml; polymyxin and nitrofurantoin from 100 to 0.19 μg/ml; and sulfadiazine from 5 to 0.009 mg/ml. The tubes were then incubated at 37 C for 24 to 48 hr and readings recorded. Of the following organisms, five clinical or standard strains of each were used in this study: *Klebsiella pneumoniae, Aerobacter aerogenes, Proteus vulgaris, Pseudomonas aeruginosa, Escherichia coli, Staphylococcus aureus,* and *Enterococcus.*

*Bactericidal action.* Young and actively growing cultures of pathogenic bacteria were suspended or emulsified in 3 ml of VoSol in a test tube. At intervals of 0, 5, 10, 15, 60, 90, and 120 min, subcultures of the mixture were streaked on blood agar plates, inoculated to tubes of broth, incubated for 24 hr, and examined to determine the presence or absence of growth. The following pathogenic bacteria were used in these studies: *P. vulgaris* Jones, *A. aerogenes* Pinkerton, *Staphylococcus aureus* Y - 1, *Streptococcus pyogenes* C203 Mv, *K. pneumoniae* 35 A 39, and *E. coli* 239.

*Fungistatic effect.* VoSol was serially diluted in tubes containing 5 ml of melted Sabouraud medium which was cooled down to 42 C. The concentrations of the drug ranged from 6.25 to 0.045%. The tubes were then slanted and solidified. The next day, they were inoculated with the following cultures: *Cryptococcus neoformans, Nocardia asteroides, Microsporum audouini, Candida albicans, Trichophyton sulfurem, T. rubrum,* and *T. mentagrophytes.* The tubes were then incubated at room temperature for two weeks and examined at the end of the first and second week of incubation to determine the presence and absence of growth.

*Fungicidal effect.* Homogenized fungal culture (0.1 ml) in saline was mixed or emulsified in a tube containing 3 ml of VoSol. At intervals of 0, 5, 10, 15, 30, 50, 90, and 120 min, subcultures were made in Sabouraud agar. The tubes were then incubated at room temperature for two weeks and readings taken at the end of the first and second week to determine the presence of growth. The following cultures of fungi were used in these studies: *C. albicans, N. asteroides, C. neoformans,* and *Phialofora pedrosi.*

## Results

Table 1 shows the bacteriostatic effect of 1,2-propanediol diacetate, VoSol, polymyxin, sulfadiazine, and nitrofurantoin. 1,2-Propanediol diacetate inhibited the growth of *K. pneumoniae* in 0.027 to 1.875 mg/ml, *A. aerogenes* in 0.117 to 7.5 mg/ml, *P. vulgaris* in 0.117 to 3.750 mg/ml, *P. aeruginosa* in 0.057 to 0.468 mg/ml, *E. coli* in 0.234 to 0.936 mg/ml, *S. aureus* in 0.117 to 3.750 mg/ml, and *Enterococcus* in 0.468 to 0.936 mg/ml. VoSol inhibited *K. pneumoniae* in 0.09

to 6.25%, *A. aerogenes* in 0.39 to 25%, *P. vulgaris* in 0.39 to 12.5%, *P. aeruginosa* in 0.19 to 1.56%, *E. coli* in 0.78 to 3.12%, *S. aureus* in 0.39 to 12.5%, and *Enterococcus* in 1.56 to 6.25%. These findings compared favorably with those of polymyxin, sulfadiazine, and nitrofurantoin.

TABLE 1. *Antibacterial spectrum of 1,2-propanediol diacetate, VoSol, polymyxin, sulfadiazine, and nitrofurantoin*

| Organism | 1,2-Propanediol diacetate | VoSol | Polymyxin | Sulfadiazine | Nitrofurantoin |
|---|---|---|---|---|---|
| | *mg/ml* | % | *μg/ml* | *mg/ml* | *μg/ml* |
| *K. pneumoniae* | | | | | |
| 803 | 0.027 | 0.09 | 0.10 | 0.009 | 0.10 |
| 55 | 0.468 | 1.56 | 0.39 | 2.5 | 100.0 |
| Ronan | 1.875 | 6.25 | 0.19 | 5.0 | 100.0 |
| 35 A 44 | 0.938 | 3.125 | 0.19 | 5.0 | 100.0 |
| Kleinzhotter | 0.027 | 0.09 | 0.39 | 0.009 | 1.56 |
| *A. aerogenes* | | | | | |
| 28 | 0.117 | 0.39 | 100.0 | 1.25 | 50.0 |
| 48 | 0.117 | 0.39 | 50.0 | 0.625 | 6.25 |
| Eros | 1.875 | 6.25 | 100.0 | 5.0 | 100.0 |
| 56 | 7.500 | 25.0 | 3.12 | 1.25 | 100.0 |
| C 17 | 0.234 | 0.78 | 0.78 | 1.25 | 100.0 |
| *P. vulgaris* | | | | | |
| Weiss | 1.875 | 6.25 | 100.0 | 1.25 | 100.0 |
| Gerstein | 3.750 | 12.5 | 3.12 | 5.0 | 100.0 |
| 28 | 0.117 | 0.39 | 100.0 | 0.009 | 100.0 |
| Sweeny | 3.750 | 12.5 | 100.0 | 5.0 | 100.0 |
| F D A 233 | 3.750 | 12.5 | 50.0 | 5.0 | 100.0 |
| *P. aeruginosa* | | | | | |
| 38 | 0.468 | 1.56 | 12.5 | 1.25 | 100.0 |
| Tyson | 0.234 | 0.78 | 12.5 | 5.0 | 100.0 |
| 34 | 0.468 | 1.56 | 12.5 | 5.0 | 6.25 |
| Higgins | 0.234 | 0.78 | 3.12 | 1.25 | 100.0 |
| 50 | 0.057 | 0.19 | 0.39 | 0.312 | 100.0 |
| *E. coli* | | | | | |
| 16 | 0.936 | 3.12 | 100.0 | 5.0 | 3.12 |
| 56 | 0.234 | 0.78 | 0.19 | 0.039 | 25.0 |
| 42 | 0.468 | 1.56 | 6.25 | 2.5 | 12.5 |
| 27 B | 0.234 | 0.78 | 100.0 | 1.25 | 100.0 |
| 23 | 0.234 | 0.78 | 1.56 | 1.25 | 6.25 |
| *S. aureus* | | | | | |
| Sudary | 3.750 | 12.5 | 100.0 | 0.625 | 12.5 |
| 46 | 0.234 | 0.78 | 6.25 | 5.0 | 1.56 |
| Light | 0.117 | 0.39 | 50.0 | 5.0 | 6.25 |
| Slezak | 0.234 | 0.78 | 50.0 | 5.0 | 12.5 |
| Von Nardhoff | 0.936 | 3.12 | 12.5 | 1.25 | 6.25 |
| *Enterococcus* | | | | | |
| 41 | 0.468 | 1.56 | 100.0 | 5.0 | 12.5 |
| Lubin | 1.875 | 6.25 | 100.0 | 5.0 | 6.25 |
| 44 | 0.468 | 1.56 | 100.0 | 5.0 | 3.12 |
| Lucas | 0.936 | 3.12 | 100.0 | 5.0 | 12.5 |
| Frank | 0.936 | 3.12 | 100.0 | 5.0 | 6.25 |

VoSol immediately killed cultures of *Klebsiella, Proteus, Pseudomonas, Aerobacter, Escherichia, Staphylococcus,* and *Enterococcus.*

Table 2 shows the fungistatic effect of VoSol. It inhibited the growth of *C. albicans, C. neoformans, M. audouini, T. rubrum,* and *T. mentagrophytes* in 3.125%, *N. asteroides* in 1.56%, and *T. sulfurum* in greater than 6.25%.

TABLE 2. *Fungistatic effect of VoSol*

| Organism | Effect |
|---|---|
| | % |
| *Cryptococcus neoformans* | 3.125 |
| *Nocardia asteroides* | 1.56 |
| *Microsporum audoini* | 3.125 |
| *Candida albicans* | 3.125 |
| *Trichophtyon sulfurum* | 6.25 |
| *Trichophyton rubrum* | 3.125 |
| *Trichophyton mentagrophytes* | 3.125 |

VoSol immediately killed cultures of *C. albicans, N. asteroides, C. neoformans,* and *P. pedrosi.*

## Discussion

Synthetic antimicrobials are less effective in vitro than antibiotics. The antibiotics exert their antibacterial effect on the pathogenic bacteria in micrograms or fractions of micrograms, whereas the antibacterials are active in milligrams. A synthetic chemical with a weak in vitro activity may, nevertheless, have a very potent in vivo effect in the animal host.

1,2-Propanediol diacetate and VoSol have an active gram-positive and gram-negative antibacterial spectrum, and are as effective in vitro as sulfonamides and nitrofurans. The antifungal activity is in the dermatophyte group as well as the group which invades the mucus membranes. This could imply that this compound may be clinically effective in skin as well as mucus membrane lesions caused by fungi. In systemic infections, the antibiotics and sulfonamides are very potent therapeutic agents, but 1,2-propanediol diacetate may prove to be very effective as a local therapeutic agent in lesions of the skin and mucus membranes. It is free of side effects, and does not induce hypersensitivity or exert toxic effects on distant organs.

# SULFONAMIDE DIFFUSION AND INHIBITION OF BACTERIAL GROWTH IN AGAR MEDIA

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The techniques in use for determining bacterial susceptibility to antimicrobial agents and the interpretation of results have recently been reviewed by an Expert Committee on Antibiotics of the World Health Organization (1961). Although this report is concerned primarily with antibiotics, much of the information is also applicable to sulfonamide susceptibility testing.

Considerable emphasis has been placed on the *p*-aminobenzoic acid (PABA) content of media for sulfonamide susceptibility testing. However, it is very probable that constituents other than PABA may also influence the performance of a medium. Syrucek and Bruckova (1958) have demonstrated significantly different in vitro susceptibilities of beta-hemolytic streptococci to sulfathiazole when the test was performed on different media. These authors divided substances which reduce or neutralize the bacteriostatic action of sulfonamides into two groups. The first included the competitive antagonists of which PABA is considered the most important. The second group included the noncompetitive antagonists which the authors feel are of greater importance than the competitive antagonists. These "indirect" antagonists were considered to be "undefinable high molecular substances, protein fragments, parts of serum and plasma, peptone, metabolites of various bacteria and some amino acid substances accelerating the growth of the bacteria." It was suggested that these noncompetitive antagonists were primarily responsible for variations in susceptibility observed on different nutrient media, rather than the more frequently cited PABA.

Several media have been proposed for sulfonamide susceptibility testing. There exist, however, divergent views on the performance of these media; Leming and Flanigan (1959) reported that none of several media demonstrated a superior performance, and Anderson and Troyanosky (1960) concluded that none of the agar base media generally used for susceptibility testing will alter zones of inhibition, leading to the erroneous interpretation of results.

This investigation was undertaken to assess some of the factors which may contribute to the performance of the disc-plate technique for demonstration of bacterial susceptibility to sulfonamides.

## Materials and Methods

*Cultures.* The bacteria employed in this study included the following (with their code numbers): *Aerobacter aerogenes* 357, 360, and 563; *Escherichia coli* 10, 14, 430, 458, 519, 430, 543, 560, 762, 778, and 779, *Klebsiella* sp. 432, 544, and 651; *Paracolobactrum* sp. 587; *Proteus* sp. 547 and 586; *Pseudomonas* sp. 276, 319, 336, 368, 436, and 661; and *Staphylococcus aureus* 1, 2, 5, 321, 335, 409, 412, 574, 598, 626, 650, 738, 887, 888, and 916. These cultures were recent isolations from clinical specimens submitted to the bacteriology laboratory of the Walter Reed General Hospital, Washington, D.C. They were selected on the basis of exhibiting varying degrees of susceptibility to sulfonamides, particularly Gantrisin (sulfisoxazole), when tested by the disc plate method with discs containing 1 mg of the drug. Cultures were maintained on cystine Trypticase Agar (Baltimore Biological Laboratory, Inc. Baltimore, Md.) (BBL).

*Susceptibility testing.* Measurement of the susceptibility of bacterial cultures to Gantrisin and other sulfonamides was accomplished by two methods, the conventional agar plate disc method and an agar technique, wherein the drug was incorporated into the medium at various concentrations prior to inoculation of the medium. The agar plate technique permitted the determination of the minimal inhibitory concentration (MIC) of the drug for each organism.

Several of the commercially available media recommended for sulfonamide susceptibility testing were used, namely Mueller Hinton agar (Difco and BBL), SR medium (Difco) supplemented with agar to a final concentration of 15 g/liter, antibiotic sulfonamide disc medium (Case 237); and Trypticase soy agar (BBL). They were evaluated for performance by observing the zone of inhibition produced by a sulfonamide-containing disc used against a susceptible organism. Comparisons were made of zone size and zone quality, i.e., sharpness of the edge of zone of inhibition, the amount of growth within the zone, and the appearance of secondary zones. The scheme used for interpretation of the quality of zones of inhibitation is illustrated in Fig. 1. At the same time, several new media formulations were prepared and also evaluated in the same manner. Of the experimentally prepared media tested, the following, in g/liter, was selected as the reference medium for the performance of all tests: Peptone (Trypticase), 10; beef extract, 10; $Na_2HPO_4$, 0.66; $KH_2PO_4$, 0.3; and agar, 15.0 (final pH 7.3). This medium is referred to as the BEP medium.

All media were dispensed in 15-ml amounts into 100 × 15 mm petri dishes which were placed on a level surface to provide a uniform thickness of medium. The inoculum for each culture consisted of a 1:10 dilution prepared from a 24-hr (35 C) Trypticase soy broth (BBL) culture. This inoculum was spread uniformly over the surface of the medium by means of a sterile cotton swab. Paper discs

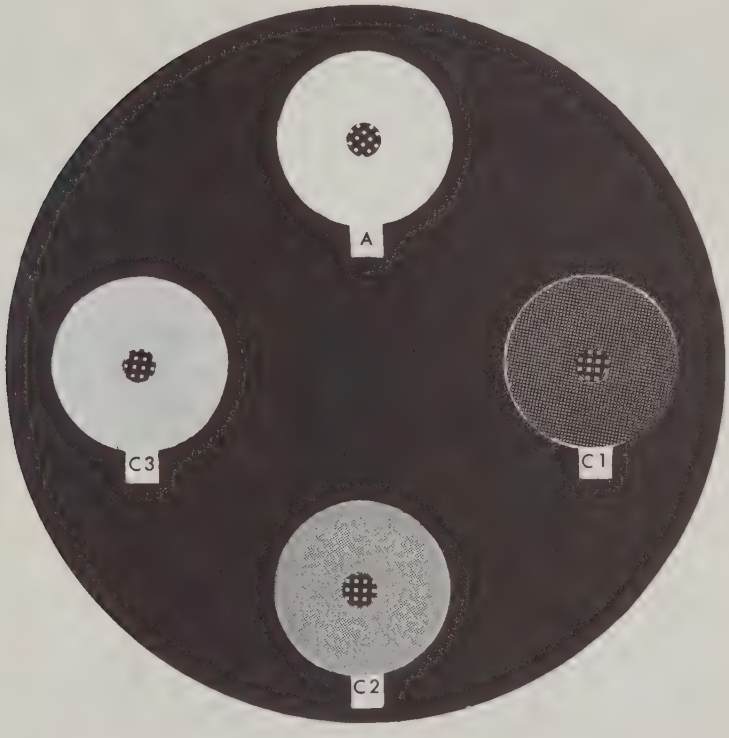


FIG. 1. *Scheme for interpreting and recording zone size and quality for sulfonamide testing. A = no visible growth within zone, sharp edge; C = visible growth within zone (C3 = very slight, C2 = slight, and C1 = moderate).*

(Schleicher and Schuell no. 740-E, 6.35 mm in diameter) containing various amounts of Gantrisin or other sulfonamides were used in the disc-plate technique. Plates were incubated at 35 C and observed at various time intervals for the appearance of growth in the agar plate technique (MIC determinations) or for zones of inhibition in the disc-plate technique.

*Measurement of diffusion of Gantrisin through media.* The diffusion of Gantrisin through the agar media was determined as follows. Discs, impregnated with known amounts of Gantrisin, were placed on plates (100 × 15 mm petri dishes) which contained 15 ml of media. Only one disc, placed in the center, was used per plate. All plates were poured on a level surface and were incubated under the same conditions as those used in susceptibility testing. At various time intervals, two duplicate plates were removed from the incubator and the Gantrisin content of the medium at various distances

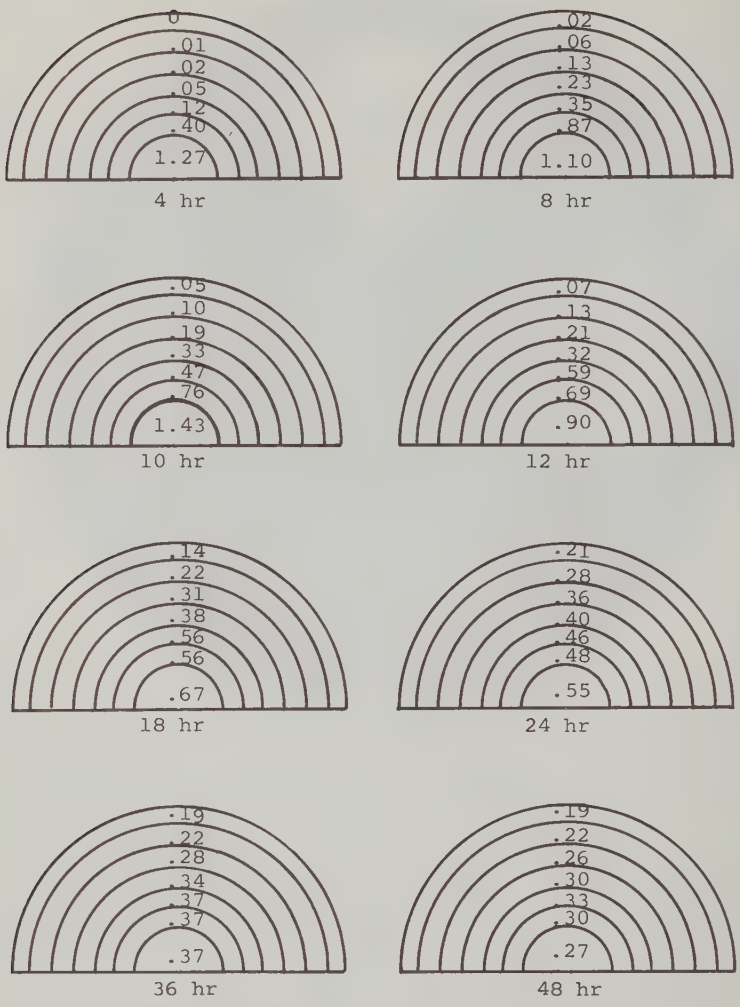


FIG. 2. *The Gantrisin content (mg/g of agar) of zones of BEP medium at various time intervals following the application of a 1.0-mg Gantrisin disc to the center of the plate. The center ring represents a distance of 8 mm from the center of the disc, each subsequent ring represents a 2-mm interval.*

from the disc was determined. Sections of the agar medium, 2 mm wide, were removed from the plates by cutting rings with a sharp pointed compass in the agar at various distances from the center of the disc. Each section represented a band 2-mm wide at various distances from the disc. These cut-out bands of medium were

weighed and their drug content was determined by the method of Bratton and Marshall, as outlined in the methods and calibrations handbook, cat. no. 33-29-40, of the Bausch and Lomb "Spectronic 20" colorimeter. From this data, the diffusion pattern of Gantrisin from a 1-mg disc was established (Fig. 2). Table 1 relates diffusion from discs containing graded amounts of drug and corresponding zones of inhibition.

TABLE 1. *Diffusion and zones of inhibition produced by various amounts of Gantrisin in BEP medium*

| Amount of Gantrisin per disc | Gantrisin concentration in medium (mg/ml) | | Zone size and quality | | |
|---|---|---|---|---|---|
| | | | Cultures | | |
| | 20 - 24–mm zone | 30 - 34–mm zone | 1 | 436 | 540 |
| *mg* | | | | | |
| 1.0 | 0.55* | 0.12 | 32C1† | 31C1 | 32C1 |
| | 0.71 | 0.44 | 43A | 20C1 | 34C3 |
| 0.5 | 0.27 | 0.08 | 34C1 | 26C1 | 32C2 |
| | 0.36 | 0.15 | 40A | 23C3 | 28C2 |
| 0.25 | 0.13 | 0.04 | 31C1 | 22C1 | 26C1 |
| | 0.20 | 0.12 | 38A | 19C1 | 25C2 |
| 0.125 | 0.07 | 0.03 | 28C1 | 21C1 | 27C1 |
| | 0.09 | 0.05 | 35A | 13C1 | 23C2 |
| 0.062 | 0.03 | 0.02 | 27C1 | 17C1 | 26C2 |
| | 0.05 | 0.04 | 30A | R | 20C3 |

*The first number is from an 8-hr observation and the second, a 24-hr observation.

†In each instance, the first number denotes the diameter of the zone of inhibition (mm), and the letter and number denote the quality of the zone of inhibition (see Fig. 1). R = reversal of inhibition.

*Reversal of sulfonamide inhibition with PABA.* Since the PABA content of media is frequently referred to as being responsible for poor results in sulfonamide susceptibility testing, studies were performed to determine the amount of PABA required to interfere with sulfonamide activity. Five different media, previously assayed for their PABA content, were supplemented with additional PABA in amounts ranging from 0.01 to 10 μg/ml. These media were streaked with several organisms exhibiting MIC's to Gantrisin from less than 0.025 μg/ml to greater than 0.2 μg/ml. Sulfonamide discs (Gantrisin or Triple Sulfa) containing 1.0, 0.25, and 0.05 mg of the drug were placed on each plate. Differences in zone sizes and zone quality were recorded at various time intervals (Table 2).

TABLE 2. *Effect of PABA on the reversal of inhibition by Triple Sulfa in the BEP medium as determined by the reduction of zone size and quality*

| Amount of PABA added to medium | 1.0 mg of Triple Sulfa per disc | | | | | 0.25 mg of Triple Sulfa per disc | | | | | 0.055 mg of Triple Sulfa per disc | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Cultures | | | | | Cultures | | | | | Cultures | | | | |
| | 1 | 432 | 436 | 540 | 547 | 1 | 432 | 436 | 540 | 547 | 1 | 432 | 436 | 540 | 547 |
| *μg/mg* | | | | | | | | | | | | | | | |
| 0 | 34C1* | 33C2 | 35C2 | 30C2 | 38C2 | 30C1 | 28C2 | 31C2 | 27C2 | 35C2 | 25C1 | 21C2 | 24C2 | 21C2 | 30C2 |
| | 32A | 30C2 | 30A | 30C2 | 32A | 27A | 26C2 | 24C3 | 24C2 | 25C3 | 16C2 | 21C1 | 18C1 | 18C1 | 23C1 |
| | 30A | 30C2 | 28C3 | 27C3 | 28A | 27A | 25C2 | 21C2 | 23C3 | 23C3 | 16C2 | 20C2 | ± | 13C2 | 17C1 |
| 0.01 | 33C1 | 29C2 | 33C2 | 30C2 | 32C2 | 27C1 | 27C2 | 28C2 | 27C2 | | 24C1 | 20C2 | 24C2 | 20C2 | 25C2 |
| | 32A | 28C2 | 29A | 28C2 | 30C3 | 25A | 24C2 | 21C3 | 22C2 | | 15C2 | 18C2 | 15C1 | 16C1 | 23C1 |
| | 30A | 27C2 | 28C3 | 28C3 | 30C3 | 25A | 25C2 | 21C2 | 23C3 | | 15C2 | 18C1 | ± | 14C2 | 15C1 |
| 0.1 | 32C1 | 28C2 | 33C2 | 30C2 | 35C2 | 27C1 | 26C2 | 27C2 | 27C2 | 32C2 | 24C1 | 20C1 | 23C2 | 19C2 | 25C2 |
| | 30A | 28C2 | 30A | 28C2 | 30C3 | 24A | 23C2 | 22C2 | 22C2 | 25C2 | 15C2 | 18C2 | 16C1 | 13C1 | 23C1 |
| | 28A | 26C2 | 29C3 | 28C3 | 30C3 | 24A | 24C2 | 21C2 | 23C3 | 20C2 | 15C2 | 18C1 | ± | 13C1 | ± |
| 1 | 28C1 | 26C2 | 33C2 | 28C2 | 33C2 | 23C1 | 22C2 | 25C2 | 22C2 | 28C2 | 15C2 | 15C1 | 22C2 | 15C1 | 21C2 |
| | 29A | 25C2 | 29A | 23C2 | 30C3 | 19A | 23C2 | 18C2 | 17C2 | 24C2 | ± | ± | ± | ± | 17C1 |
| | 27A | 26C2 | 28C3 | 26C3 | 30C3 | 19A | 22C1 | 19C1 | 17C1 | 20C2 | ± | ± | − | ± | ± |
| 10 | 22C1 | 21C2 | 27C2 | 21C1 | 25C2 | 15C1 | 18C1 | 20C2 | 17C1 | 23C2 | − | ± | 15C1 | ± | 12C1 |
| | 22A | 20C2 | 23A | 16C2 | 22C3 | ± | 13C1 | ± | ± | 17C3 | − | − | − | − | ± |
| | 20A | 18C2 | 21C3 | 17C1 | 21C3 | ± | 12C1 | − | ± | 17C3 | − | − | − | − | ± |

*The three readings refer to 8-, 24- and 48-hr observations. In each instance, the first number denotes diameter of inhibition zone, and the letter and number denote quality of inhibition zone (see Fig. 1); (±) and (−) refer to a questionable and no zone, respectively.

## Results and Discussion

*Diffusion of Gantrisin through agar media.* The diffusion pattern of Gantrisin from a paper disc through agar is shown in Fig. 2. It should be noted that the figure given for the concentration of drug in each 2-mm band represents the average of the gradient concentration existing within this band of medium. The time at which the most uniform gradient of concentration (from the center of the disc to the edge of the petri dish) appeared to be in the vicinity of 8 to 10 hr. Hence, as will become evident from data presented later, inhibition response (size of the zone of inhibition) determined at this time of incubation is most nearly correlated with the MIC of the organism.

The diffusion of Gantrisin from paper discs impregnated with various amounts of the drug through BEP medium and other media recommended for susceptibility testing of sulfonamides was compared. In this series of experiments, only two bands of the agar medium were assayed for drug content: namely, a band 2-mm wide beginning 10 mm from the center of the disc and another 2-mm band beginning 15 mm from the center of the disc. The results presented in Table 1 are those obtained with the BEP medium. The diffusion of the drug in several other media was very similar to that shown for the BEP medium. However, there were differences in the size and quality of the zones produced on the different media with the same organism. For example, a zone of 31 mm was produced after 8 hr on the BEP medium with culture 436, although on Mueller Hinton agar a zone of only 23 mm was produced. These differences were not related to drug diffusion but to the effect of the medium on the growth of the organism. The media permitting more rapid bacterial growth tend to yield smaller and poorer quality zones of inhibition, and the nutritionally poorer media yield larger zones of better quality. Furthermore, organisms which have a short generation time will produce macroscopically detectable growth on short incubation. Therefore, zones of inhibition will become evident earlier with the more rapidly growing organisms and the zones will be smaller since the drug has not had sufficient time to diffuse further into the medium and establish a concentration equivalent to the MIC. Since the inhibitory properties of sulfonamides are not manifested until initial bacterial growth has occurred, the extent of that growth will determine the quality of the zone of inhibition. It should be noted that the amount of growth will be influenced by the size of the inoculum.

*Correlation of zone size with MIC.* Further studies were performed to relate the information on drug diffusion through agar media to the sizes of zones of inhibition, employing tne conventional disc plate technique and cultures of known MIC's. These values were determined by incorporating Gantrisin in the BEP medium to give the

TABLE 3. *The comparison of actual zone size with the predicted zone size on the basis of MIC and the Gantrisin content of various areas of the BEP medium*

| Cultures | MIC of Gantrisin | Predicted zone size (mm) | | | | | Actual zone size (mm)* | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | Incubation time | | | | | Incubation time | | | | |
| | | 4 hr | 8 hr | 10 hr | 12 hr | 18 hr | 4 hr | 8 hr | 10 hr | 12 hr | 18 hr |
| | *mg/ml* | | | | | | | | | | |
| 1 | <.025 | 24-28 | 36-40 | 36-40 | 36-40 | 36-40 | – | 38 | 39 | 40 | 40 |
| 2 | <.025 | 24-28 | 36-40 | 36-40 | 36-40 | 36-40 | – | 40 | 40 | 40 | 38 |
| 5 | <.025 | 24-28 | 36-40 | 36-40 | 36-40 | 36-40 | – | 37 | 37 | 38 | 40 |
| 10 | <.025 | 24-28 | 36-40 | 36-40 | 36-40 | 36-40 | – | 36 | 36 | 35 | 32 |
| 14 | <.025 | 24-28 | 36-40 | 36-40 | 36-40 | 36-40 | – | 36 | 37 | 38 | 37 |
| 276 | >0.1 <0.2 | 16-20 | 24-28 | 24-28 | 28-32 | 32-36 | – | 27 | 29 | 28 | 23 |
| 319 | >0.2 <0.4 | 16-20 | 20-24 | 20-24 | 20-24 | 24-28 | – | ±(35) | 32 | 30 | 21 |
| 321 | <.025 | 24-28 | 36-40 | 36-40 | 36-40 | 36-40 | – | 40 | 39 | 38 | 40 |
| 335 | <.025 | 24-28 | 36-40 | 36-40 | 36-40 | 36-40 | – | 40 | 45 | 45 | 41 |
| 336 | >025<.05 | 24-28 | 32-36 | 32-36 | 36-40 | 36-40 | – | 30 | 28 | 29 | 30 |
| 357 | >.025<.05 | 24-28 | 32-36 | 32-36 | 36-40 | 36-40 | – | 31 | 31 | 31 | 33 |
| 360 | <.025 | 24-28 | 36-40 | 36-40 | 36-40 | 36-40 | – | 38 | 40 | 41 | 41 |
| 368 | >0.2 <0.4 | 16-20 | 20-24 | 20-24 | 20-24 | 24-28 | – | 26 | 25 | 24 | 21 |
| 409 | <.025 | 24-28 | 36-40 | 36-40 | 36-40 | 36-40 | – | 39 | 40 | 39 | 39 |
| 412 | <.025 | 24-28 | 36-40 | 36-40 | 36-40 | 36-40 | – | 38 | 39 | 38 | 38 |
| 430 | <.025<.05 | 24-28 | 32-36 | 32-36 | 36-40 | 36-40 | – | 34 | 32 | 31 | 30 |
| 432 | >0.1 <0.2 | 16-20 | 24-28 | 24-28 | 28-32 | 32-36 | – | 32 | 33 | 32 | 33 |
| 436 | >0.2 <0.4 | 16-20 | 20-24 | 20-24 | 20-24 | 24-28 | – | 24 | 23 | 22 | 20 |
| 547 | <.025 | 24-28 | 36-40 | 36-40 | 36-40 | 36-40 | – | 37 | 40 | 39 | 37 |
| 888 | <.025 | 24-28 | 36-40 | 36-40 | 36-40 | 36-40 | – | 41 | 40 | 39 | 39 |
| 916 | <.025 | 24-28 | 36-40 | 36-40 | 36-40 | 36-40 | – | 39 | 40 | 40 | 39 |

*Diameter of zone was produced by discs containing 1 mg Gantrisin.

following drug content in mg/ml of final medium: 0.4, 0.2, 0.1, 0.05, 0.025, and 0. The lowest concentration inhibiting growth following streak inoculation was considered the MIC. In many cases, the medium containing an amount of drug recorded as the MIC was not completely free of growth, but the next higher concentrations of the drug failed to further decrease this amount of growth. Koopmans (1960) has reported that microcolonies of 30 to 50 cells were formed after 3 to 4 hr, regardless of the susceptibility of the organism or the drug content of the medium.

Having established the MIC for a culture in an agar medium and knowing the diffusion pattern of the drug from a disc through this medium, it follows that one should be able to predict beforehand the size of the zone of inhibition that would be produced by such a disc against this organism (Table 3). The predicted zone size was reached by relating the MIC value for each organism to the data shown in Fig. 2 to locate the area of the plate at each incubation time, which would contain the drug in a concentration equal to or greater than the MIC.

The number of bacterial cells contained in the inoculum and the incubation period (time at which zones are measured) are of paramount importance when the MIC of a sulfonamide is determined from the diameter of a zone of inhibition. The zone diameter must be measured at the earliest possible time, usually about 8 hr, to achieve

a good correlation between MIC and zone size. The inoculum should be sufficient to give visible growth within 8 to 10 hr, at which time a uniform gradient of concentration of the drug exists in the medium surrounding the disc (Fig. 2). Too large an inoculum will obscure the effect of the drug, as visible growth appears before inhibition is manifested by the drug. Once the inhibition zone is formed, there is little change in its size within the next few hours, although there is a considerable change in the drug content in the area farther from the disc. Therefore, the earliest time at which the initial inhibitory zone can be measured will provide data which give the best correlation between the MIC and zone size. It is seen that there was insufficient growth to observe inhibition after 4 hr, but after 8 hr there was sufficient growth to measure zones (Table 3). With a few exceptions, the organisms gave the predicted responses at the 8- and 10-hr incubation periods. Differences of 1 or 2 mm between actual and predicted zone sizes were not considered significant, since this would fall within the experimental error. After 12 hr of incubation, there was further diffusion of the drug, resulting in inhibitory concentrations being established farther from the disc (Fig. 2). Theoretically the zones of some of the more resistant organisms (for example, cultures 276, 319, 368, 432, and 436) should have become larger. However, since abundant growth had occurred in these areas prior to the time (12 hr) at which an inhibitory concentration of the drug had diffused to these areas, there could not have been an increase in zone size. Thus, there is usually no increase in zone size once growth becomes evident. In fact, with the more resistant organisms, a decrease in zone size may occur. For example, the pseudomonad designated as 368 showed a decrease in zone size of 5 mm in 10 hr. This may be attributed to the synthesis of sulfonamide antagonists by the organism; as the sulfonamides are bacteriostatic in nature, such antagonists could overcome the inhibitory effects of the drug, thus permitting growth in the area of the original zone of inhibition.

The MIC's of 20 additional cultures were predicted from the size of the zone of inhibition and compared to the MIC's determined experimentally by the agar plate method. Good correlation between predicted and actual MIC's was obtained. Thus, once the diffusion pattern of a drug has been established, zone sizes can be correlated with MIC's under carefully controlled experimental conditions.

*Effect of supplementing media with PABA on sulfonamide susceptibility testing.* The standard explanation for differences in performance of media used in sulfonamide susceptibility testing usually includes some vague generalizations on the PABA content of the media. The poorer performing media supposedly have a higher PABA content and, thus, are capable of neutralizing the sulfonamide inhibition. To assess the significance of PABA in the disc-plate technique for sulfonamide susceptibility testing, each of several media were

supplemented with additional pure PABA (Table 2). Five organisms were selected, representing varying degrees of susceptibility to sulfonamides, and these were tested by the disc-plate procedure on each medium, using discs containing three different amounts of Triple Sulfa and Gantrisin. The plates were observed after 8, 24, and 48 hr of incubation for the size and quality of each inhibition zone. Primary interest was centered on any suppression of inhibitory effect by the media containing additional PABA.

Detailed data (Table 2) are presented for only the BEP medium, although the same information was tabulated for each of the different media used. In no instance did a disc, containing 1 mg of either sulfonamide, fail to give a zone of inhibition, even on the media supplemented with 10 $\mu$g/ml of PABA. However, there was a reduction in zone size as the PABA content of the medium increased. The results obtained with the discs containing 0.25 mg of either sulfonamide indicate that the PABA content of a medium must exceed 1 $\mu$g/ml to completely reverse inhibition. There was a decrease in the size of the zones produced on the media containing 1 $\mu$g/ml of PABA, but a zone of inhibition could be clearly distinguished even after 48 hr of incubation. In most instances, the addition of 1 $\mu$g/ml of PABA to the media sufficiently reduced the size and the quality of the zones of inhibition, produced by a disc containing 0.05 mg of sulfonamide, to obscure inhibition after 24 hr. The media containing up to 0.1 $\mu$g/ml of added PABA gave only a slight reduction of zone size or quality when a 0.05-mg disc was used. Recently, Park (1961) assayed several media, recommended for testing the susceptibility of bacteria to sulfonamides, for their PABA content, and found from 0.004 to 0.056 $\mu$g/ml. The level of PABA necessary to significantly alter inhibition zones produced by a disc containing as little as 0.05 mg of Triple Sulfa (or Gantrisin) exceeds 0.1 $\mu$g/ml (Table 2 and Fig. 3). Therefore, it must be concluded that the PABA content of the media employed in this study is not sufficiently high to influence bacterial inhibition by sulfonamides.

The evidence presented here show a correlation between the diffusion pattern of a drug, the zone of inhibition produced by a disc containing that drug, and the MIC for an organism. These data require the performance of the test under carefully controlled conditions. Specifically, there must be a standardization of the inoculum and the reading of the results after a prescribed incubation period. It must be recognized that this may not be possible in the routine diagnostic bacteriology laboratory. Therefore, only a qualitative estimation of the susceptibility of the organisms to sulfonamides can be made, if strict adherence to standardized techniques is not practical.

The results indicate that the disc-plate procedure can be performed in a manner to provide satisfactory information as to the in vitro susceptibility of bacteria to sulfonamides. However, as stated

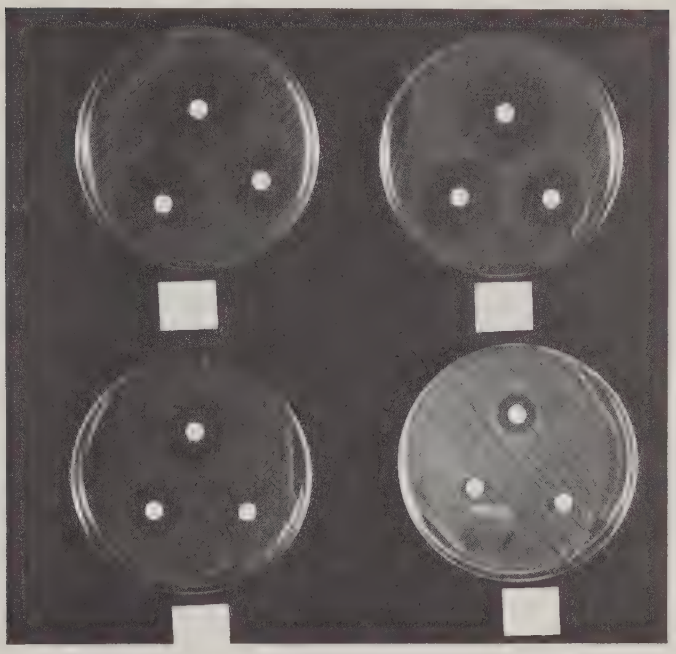

FIG. 3. *The influence of PABA on the size and quality of zones of inhibition produced by Gantrisin using the BEP medium. The concentration (μg/ml of medium) of PABA added to the medium is: plate 1 = 0, plate 2 = 0.1, plate 3 = 1.0, and plate 4 = 10. The Gantrisin content of the discs is: 1.0 mg (largest zone), 0.25 mg, and 0.05 mg (smallest zone).*

by Gould (1960), "The value of all laboratory sensitivity tests must be the correlation between their results and those in the patient receiving specific therapy. There is general agreement that these tests are of value to the clinician but there is a paucity of factual information, with the result that we are as yet at an elementary stage in correlating the clinical administration of antibiotics with in vitro sensitivity determinations designed to give some idea of the amount of antibiotic required."

This aspect of susceptibility testing has also been discussed recently by Greenberg (1960).

Acknowledgment

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## Literature Cited

Anderson, T. G., and A. Troyanosky. 1960. Antibiotic susceptibility testing by the disc method. Antibiotics Ann. 1959-1960, p. 587-595.

Gould, J. C. 1960. The laboratory control of antibiotic therapy. Brit. Med. Bull. **16**:29-34.

Greenberg, L. 1960. Problems concerned with clinical laboratory testing and the use of antibiotics. Can. Med. Serv. J. **16**:392-396.

Koopmans, R. K. 1960. A new, simple, quantitative micromethod for the rapid determination of bacterial susceptibility. Antibiotics & Chemotherapy **10**:612-622.

Leming, B. H., and C. Flanigan, Jr. 1959. Comparative sensitivity of pathogenic bacteria to modern antibacterial agents. Ann. N. Y. Acad. Sci. **82**:34-39.

Park, C. H. 1961. The microbiological assay of p-aminobenzoic acid with *Acetobacter suboxydans* and *Neurospora crassa*. M.S. Thesis, University of Maryland, College Park, Md.

Syrucek, L., and M. Bruckova. 1958. Notes on the determination of sulphonamide resistance in beta haemolytic streptococci *in vitro*. J. Hyg. Epidemiol. Microbiol. Immunol. **2**:155-171.

World Health Organization. Expert Committee on Antibiotics. 1961. Standardization of methods for conducting microbic sensitivity tests. Technical report series, no. 210.

# A SERIES OF SYNTHETIC ANTIMICROBIAL COMPOUNDS
## I. Chemistry

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In the second paper of this series are the results of a screening program in which approximately 200 compounds were tested against 21 microorganisms. The purpose of this report is to discuss concepts which led to the preparation of these materials and to relate either chemical structure or chemical behavior to antimicrobial activity.

### Materials and Methods

The compounds under discussion are derived from the syntheses shown in Fig. 1. This figure illustrates the reaction of a carbonyl compound with a carbonyl reagent; the splitting out of water results

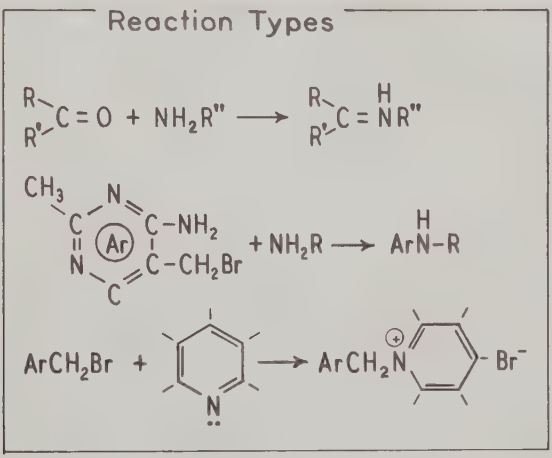


FIG. 1. *Derivation of antimicrobial compounds using carbonyl compounds.*

in a stable compound. The carbonyl compounds used in the present studies include aldehydes, ketones, esters, and nitriles. The reaction proceeds in either aqueous, alcoholic, or mixed systems. Adducts are obtained by concentrating the reaction mixture. Crystallization occurs upon the addition of a nonpolar solvent such as petroleum ether. Figure 1 also shows the reaction of a bromomethyl pyrimidine with a primary or tertiary amine yielding either a secondary amine or a quaternary ammonium salt. The alkylation is run in either butyl alcohol or toluene, depending largely on the solubility properties of the amine used. These compounds are included in the present study because they are structural analogues of pteroic acid and, in some instances, thiamine. They are related chemically to aldehyde adducts which have been reduced. They are analogues of compounds whose biosyntheses proceed by way of Schiff base formation.

Domagk et al. (1946) indicated that thiosemicarbazones derived from acetamidobenzaldehyde and thiosemicarbazide, or para-dimethylaminobenzaldehyde and the same carbonyl reagent, possessed significant antitubercular activity. They also noted that some related compounds possessed either anti-inflammatory or analgetic activity.

Fox (1952) initially aimed at preparing isosteres of Domagk's compounds by the insertion of the nitrogen of aminobenzaldehyde into an aromatic ring to give a pyridine aldehyde. Isonicotinic hydrazide was an intermediate in his synthesis of the pyridine-4-aldehyde thiosemicarbazone, and the decision to evaluate this intermediate for antitubercular activity has contributed greatly to present day chemotherapy of tuberculosis. As Domagk's compounds gave rise to anti-inflammatory drugs, the compounds of Fox have given rise to analeptics.

A number of investigators have demonstrated antagonism between isonicotinic hydrazide and pyridoxal in growth experiments involving microorganisms and mammals. It was shown that pyridoxal can interfere with the convulsive episodes induced by isonicotinic hydrazide. However, efforts to demonstrate an antagonism between the two substances in pyridoxal-containing enzymes such as transaminase were not successful. McCormick and Snell (1959) were the first to show an effect of hydrazides on enzymes in a cell-free system. They demonstrated inhibition of pyridoxal kinase, the enzyme which phosphorylates pyridoxal to its active coenzyme form by carbonyl reagents and adducts of such reagents with carbonyl compounds. They were further able to demonstrate the presence of the pyridoxal adduct of the carbonyl agent in an enzymatic system, indicating that transfer of a carbonyl reagent from its adduct to another carbonyl compound is possible. The adducts of pyridoxal and carbonyl reagents such as hydroxylamine are extremely powerful poisons for the pyridoxal kinase. In a portion of our study a number of aldehydes, which are structurally related to pyridoxal, were used as reactants

for the preparation of adducts. These include the pyridine, quinoline, and aniline analogues. Their structural relation to pyridoxal is shown in Fig. 2.

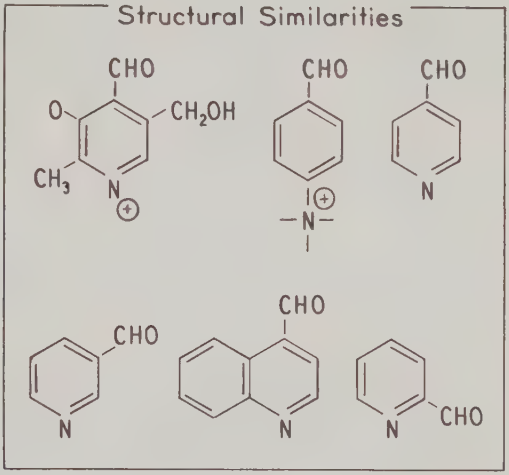


FIG. 2. *Structural relationship between aldehydes and pyridoxal.*

We have taken a compound of known activity such as isonicotinic hydrazide or *p*-dimethylaminobenzaldehyde thiosemicarbozone and attempted to discern if its antimicrobial activity is due primarily to its carbonyl component, its hydrazine portion, or to the whole molecule. We have discussed structural analogues of the pyridine moiety, and considered that, in some manner, poisoning of pyridoxal enzymes is involved in the antimicrobial activity of these compounds.

Another relation in carbonyl compounds is the relative chemical activity of adducts. The structural characteristics of two carbonyl compounds may be so different that they cannot be considered as structural analogues. However, the carbonyl functions may have almost identical chemical activity. It is conceivable that where the first compound is a natural metabolite the second one may act in a competitive way for either enzyme or another substrate (Fig. 3).

For diagrammatic purposes, the chemical reactivity of the carbonyl functions have been arranged in an ascending series (Fig. 3). Benzaldehyde has arbitrarily been assigned to a value of 4 so that less reactive carbonyls would always be considered as positive. Note that alkyl pyridinium aldehydes (such as pyridoxal) have carbonyl activity greater than benzaldehyde. The structurally similar pyridine aldehyde has carbonyl activity less than that of benzaldehyde.

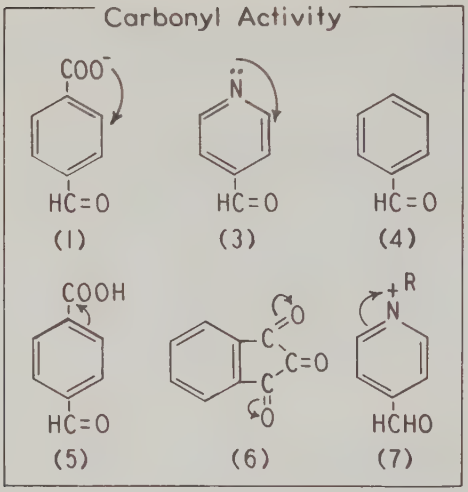


FIG. 3. *Chemical activity of carbonyl compounds.*

The structurally unrelated compound, ninhydrin, reacts in a manner similar to that of pyridoxal (Fig. 4). Whether a compound, which behaves chemically in a manner similar to a natural product, acts as an antagonist of the natural product is a matter which requires further exploration.

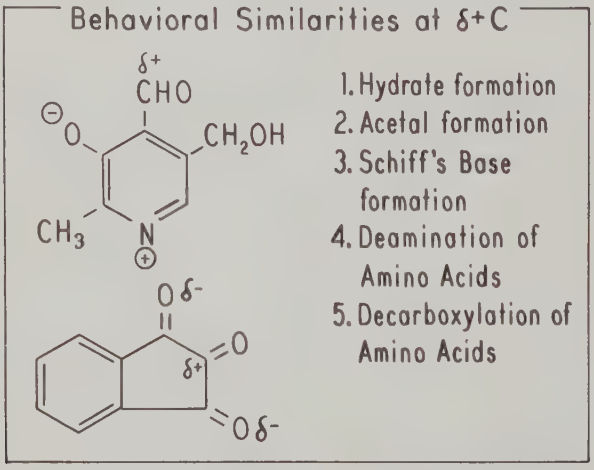


FIG. 4. *Activity of ninhydrin and pyridoxal.*

The reactions shown in Fig. 5 may be of value in understanding the antibacterial behavior of certain adducts. Upon entry into the cell, the compound may be hydrolyzed according to the first equation. This would assign to the adduct the function of being simply a carrier for a carbonyl reagent or the carbonyl compound. How easily the hydrolysis occurs would, of course, be a reflection of the polarity of the parent carbonyl function. The liberated carbonyl compound would then be competing with native substrates for the carbonyl reagent.

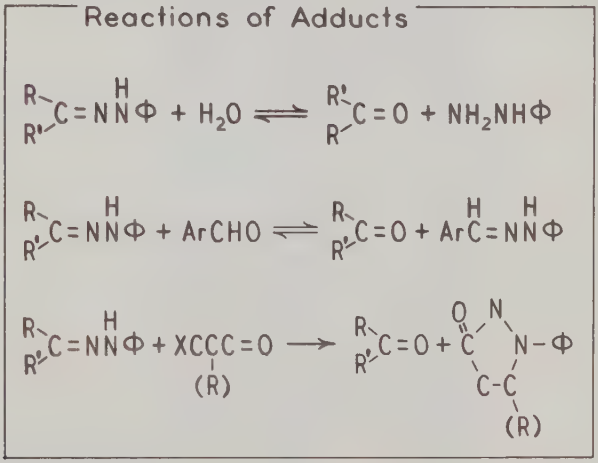


FIG. 5. *Antimicrobial activity shown by reactions of adducts.*

The second reaction indicates a more probable fate. Here the adduct, having arrived in the cell, reacts with a more reactive aldehyde, such as pyridoxal, in a purely reversible manner. One of the products is a poisoned native system.

The third reaction is a situation leading to the formation of a compound so stable that the reaction may be considered as an irreversibly poisoning one. This is the only reaction which places emphasis on the nature of the entire molecule entering the cell, and especially the reactivity of the carbonyl reagent.

Figure 6 diagrams the reactants used to prepare the adducts used in the microbiological testing.

## Discussion

Several general relations are shown in the compounds studied. For general antimicrobial activity, compounds structurally related to

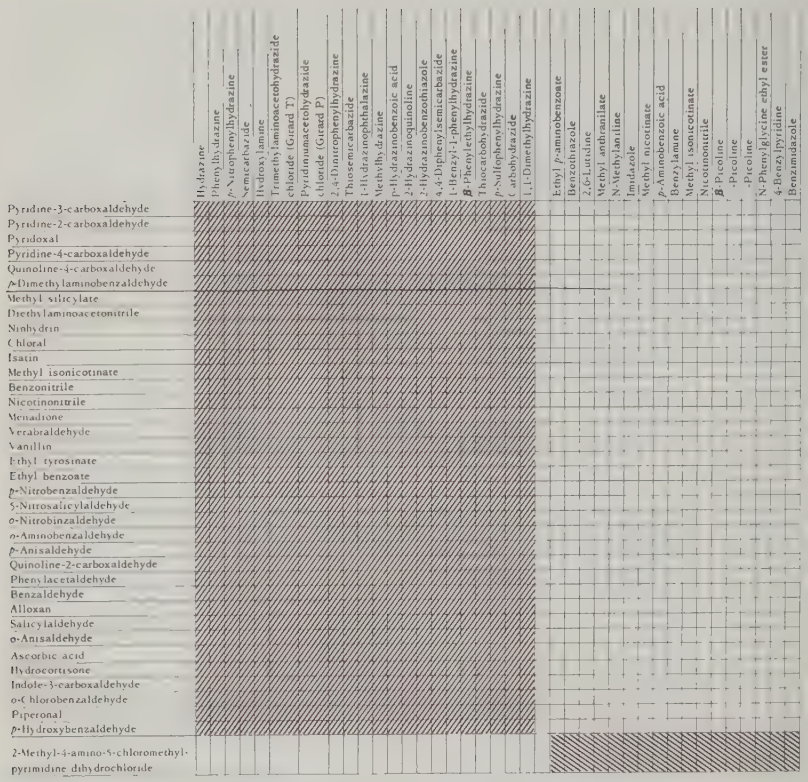


FIG. 6. *Reactants used to prepare adducts for microbiological testing.*

pyridoxal and vitamin K were most active. All of these could be expected to revert and yield the free carbonyl inside the cell. In activity against actinomycetes a different pattern is discernible. Here, activity is restricted to carbonyls having the same polarization as pyridoxal but with a structure not related to the vitamin. For general antimicrobial activity the most useful carbonyl reagents are those possessing ring structures which act as electron donors to the reactive nitrogen. This effect is not seen in activity against the mycobacteria. The rather poor activity of pyrimidyl derivatives may be related to a type of scission which is known to occur in some microorganisms (Sealock and Davis, 1949). Pyrimidine-$CH_2NH$ Ar + $H_2O$ $\longrightarrow$ pyrimidine-$CH_2OH$ + Ar $NH_2$. The pyrimidine can then be incorporated into thiamine.

## Literature Cited

Domagk, G., R. Behnisch, F. Mietzsch, and H. Schmidt. 1946. Über eine neue, gegen Tuberkelbazillen in vitro wirksame Verbindungsklasse. Naturwissenschaften **33**:315.

Fox, H. H. 1952. Synthetic tuberculostats. I. Pyridine carboxylic acid derivatives. J. Org. Chem. **17**:542-546.

McCormick, D. B., and E. E. Snell. 1959. Pyridoxal kinase of human brain and its inhibition by hydrazine derivatives. Proc. Natl. Acad. Sci. U. S. **45**:1371-1379.

Sealock, R. R., and N. C. Davis. 1949. Activating effect of *m*-nitroaniline on thiamine destruction by the Chastek paralysis enzyme. J. Biol. Chem. **177**:987-988.

# A SERIES OF SYNTHETIC ANTIMICROBIAL COMPOUNDS
## II. Antimicrobial Activity

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The approach to chemotherapy using antimetabolites has not been as successful as the random pursuit of antibiotic-producing microorganisms (Woodruff and McDaniel, 1958). The widespread clinical application of antibiotics during the last decade has resulted in the apparent selection of potentially pathogenic microorganisms not very responsive to antibiotic agents (Finland, 1960). The designing of antimetabolites, advocated by Woolley (1958), Woods and Tucker (1958), and many others and based on the rapidly mounting knowledge of biochemical differences between hosts and parasites, must again be considered promising from the viewpoint of effective therapy and selective toxicity (Albert, 1960). Therefore, the microorganisms selected to study the antimetabolic activity of the carbonyl adducts presented in the preceding paper (Seifter, Henson, and Isenberg, 1962) represent those organisms which have not been curtailed dramatically by antibiotic therapy. The preliminary study of their response to these synthetic compounds is the subject of this report.

### Materials and Methods

All bacteria, including *Mycobacterium tuberculosis*, were recent clinical isolates. The nocardiae and fungi were kindly supplied by M. Littman, Dept. of Microbiology, Mt. Sinai Hospital, New York, or obtained from the American Type Culture Collection. The microorganisms were maintained on Eugon (Baltimore Biological Laboratory) agar slants, with *M. tuberculosis* maintained on Lowenstein-Jensen medium. The susceptibility tests were carried out on Oxoid sensitivity agar (Consolidated Laboratories, Chicago Heights, Ill.), again with the exception of *M. tuberculosis* which was tested on Lowenstein-Jensen medium. Uniform paper discs (6.35 mm in diameter) were impregnated with 20 $\mu$g of ethanolic (95%) solutions of

the compounds; this volume contained 50 μg of the adducts. To yield confluent growth, the discs were then applied to sensitivity agar inoculated previously with the organisms. All the precautions advocated by Grove and Randall (1955) were observed scrupulously. Plain discs and discs containing only ethanol served as controls. Results were determined after incubation at a suitable temperature when control plates exhibited confluent growth. The zones of inhibition were measured from the periphery of the disc to the area of microbial growth with a simple apparatus, previously described (Isenberg and Shapiro, 1958).

## Results and Discussion

Table 1 summarizes the effect of the carbonyl adducts on the 21 microorganisms tested. The reaction of the organisms to the compounds was classified according to the zones of inhibition surrounding the discs impregnated with the antimicrobial compounds.

TABLE 1. *Summary of the microbial response to the carbonyl adducts*

| Organism | Response to adducts (by size of zone of inhibition) | | | Resistant forms in zone of inhibition |
|---|---|---|---|---|
| | Resistant 0-5 mm | Moderate 5-10 mm | Good 10 mm or more | |
| *Escherichia coli* | 57 | 71 | 44 | 20 |
| *Klebsiella* sp. | 72 | 45 | 45 | 30 |
| *Pseudomonas aeruginosa* | 80 | 61 | 29 | 22 |
| *Alcaligenes faecalis* | 97 | 34 | 34 | 27 |
| *Staphylococcus aureus* 80/81 | 40 | 41 | 27 | 84 |
| Enterococcus sp. | 19 | 69 | 68 | 36 |
| *Mycobacterium tuberculosis* | 171 | 17 | 4 | 0 |
| *Nocardia asteroides* | 111 | 57 | 21 | 3 |
| *Nocardia brasiliensis** | 15 | 55 | 20 | 0 |
| *Candida albicans* | 42 | 28 | 63 | 59 |
| *Cryptococcus neoformans* | 124 | 25 | 41 | 2 |
| *Coccidioides immitis* | 145 | 26 | 20 | 1 |
| *Blastomyces brasiliensis*† | 99 | 0 | 2 | 1 |
| *Blastomyces dermatitidis* | 116 | 40 | 36 | 0 |
| *Philaphorum (Hormodendrum) pedrosi* | 142 | 41 | 8 | 1 |
| *Madurella grisea*† | 100 | 0 | 2 | 0 |
| *Sporotrichum schenckii* | 149 | 35 | 7 | 1 |
| *Microsporum gypseum* | 85 | 46 | 50 | 11 |
| *Microsporum canis*‡ | 100 | 4 | 6 | 42 |
| *Microsporum audouini*‡ | 119 | 12 | 10 | 11 |
| *Trychophyton mentagrophytes*‡ | 139 | 3 | 7 | 3 |

*Tested against 90 compounds only.
†Tested against 102 compounds only.
‡Tested against 152 compounds only.

The comparison of these zones is meaningful only because the aqueous solubility of the carbonyl adducts is approximately of the same order of magnitude. Since 50 μg were used for impregnation, zones of inhibition measuring less than 5 mm were interpreted to indicate resistance to the compound. Zones between 5 to 10 mm were considered arbitrarily as representing a moderate response, and measurements above 10 mm indicated good antimicrobial activity. Actually, the limitation of space and time will permit only the presentation of those compounds which resulted in zones measuring in excess of 10 mm, and many times this includes only those which displayed zones in excess of 15 mm. The detection of isolated colonies of the organisms within the zones of inhibition led to their classification as mutants, which were placed into a distinct category. Table 1 indicates that the response of the 21 organisms to these synthetic compounds varies considerably. Although some, such as *Candida albicans*, Enterococcus sp., *Microsporum gypseum*, *Escherichia coli*, and *Klebsiella*, manifested susceptibility to one-fifth and more of the compounds, others, such as *M. tuberculosis*, *Madurella grisea*, *Blastomyces brasiliensis*, *M. canis*, and *M. audouini*, were curtailed by relatively few of the carbonyl adducts. Similarly, resistance as well as the selection of resistant variants was manifested on an individual basis.

Although this individuality of response to the carbonyl adducts is true in general, a number of compounds displayed activity against more than one of the species examined. Thus, 32 of the 192 compounds were inhibitory to five or more of the organisms tested; 24 compounds showed activity against more than five organisms, and five compounds were active against more than 10 organisms. The three adducts with the greatest general antimicrobial activity are shown in Table 2. The response to the menadione analogue is outstanding not only because 15 of the 20 organisms exposed to it responded, but also because the response was in general quite marked. This compound certainly merits investigation as a tool for the mode of action of vitamin K in physiological processes. The two additional menadione analogues synthesized with carbonyl reagents displayed neither general antimicrobial properties or sizable zones of inhibition. The pyridine-4-aldehyde derivatives, analogues of pyridoxal, were tested in many other combinations. Although certain of them displayed appreciable activity against individual or several microorganisms, the over-all antimicrobial activity displayed by these 2 substituted hydrazine adducts was not elicited by other substitutions. Pyridine-4-aldehyde phenylhydrazone displayed moderate activity against *M. tuberculosis*, the only one of the three active adducts which did so. Seifter, Henson, and Isenberg (1962) have already acknowledged that this observation is not noted for the first time in this study. The most noteworthy aspect of the three compounds is their pronounced activity against yeast and fungi. Although *Candida*

and *Sporotrichum* were resistant to pyridine-4-aldehyde benzyl phenylhydrazone, they responded to the phenylhydrazine derivative of this carbonyl compound. The menadione derivative was somewhat less effective against the dermatophytes but was quite useful in curtailing the fungal growth usually implicated in the systemic mycoses.

TABLE 2. *Compounds with general antimicrobial action*

| Organism | Menadione + diphenyl semicarbazide | Pyridine-4-aldehyde + benzyl phenyl hydrazine | Pyridine-4-aldehyde + phenyl hydrazine |
|---|---|---|---|
| *Escherichia coli* | 10* | 12 | 10 |
| *Klebsiella* sp. | 12 | 12 | R |
| *Pseudomonas aeruginosa* | 8 | R | 10 |
| *Alcaligenes faecalis* | 15 | R | 11 |
| *Staphylococcus* 80/81 | 27 | 8 | 10 |
| Enterococcus sp. | 10 | 13 | 9 |
| *Mycobacterium tuberculosis* | R | R | 9 |
| *Nocardia asteroides* | 20 | 15 | 10 |
| *Nocardia brasiliensis* | X | X | 13 |
| *Candida albicans* | 23 | R | 12 |
| *Cryptococcus neoformans* | 45 | 29 | 12 |
| *Coccidioides immitis* | 24 | 19 | 14 |
| *Blastomyces brasiliensis* | 27 | 17 | X |
| *Blastomyces dermatitidis* | 19 | 33 | 10 |
| *Philaphorum pedrosi* | R | 22 | 14 |
| *Madurella grisea* | 25 | 12 | X |
| *Microsporum gypseum* | 16 | 36 | 23 |
| *Microsporum canis* | M(17) | 22 | 11 |
| *Microsporum audouini* | 39 | 20 | 30 |
| *Sporotrichum schenckii* | R | R | 8 |
| *Trychophyton mentagrophytes* | M(26) | 8 | 12 |

*Numbers indicate radial zones of inhibition in mm; X = test not performed; R = resistant; M= mutants present, zone size in parentheses.

Table 3 is concerned with those adducts which displayed good antibacterial action in this in vitro assay. Compounds were included only if they resulted in zones of inhibition of 15 mm or more against at least one of the test organisms. The most striking feature of this table is that only four compounds, quinoline-4-aldehyde-phenylethylhydrazone, pyridoxalthiosemicarbazone, benzamidoxime (the reaction product of benzonitrile and hydroxylamine), and menadione-diphenyl semicarbazone, were active against all the bacteria tested, although not always with zones of inhibition greater than 10 mm. It appears that these carbonyl adducts underline the differences between such comparatively close relatives as *E. coli* and *Klebsiella*. The high degree of resistance by *Klebsiella* and *Pseudomonas* against chemotherapeutic agents was already shown (Table 1). Both did not respond adequately in approximately 50% of the tests. The response of these organisms to those adducts with antibacterial properties was

TABLE 3. *Compounds with good antibacterial activity*

| Carbonyl compound | Carbonyl reagent | Organisms | | | | | |
|---|---|---|---|---|---|---|---|
| | | *E. coli* | *Klebsiella* sp. | *P. aeruginosa* | *A. faecalis* | *S. aureus* 80/81 | Enterococcus sp. |
| Quinoline-4-aldehyde | Phenylethyl hydrazine | 16* | 8 | 13 | 8 | 13 | 12 |
| Pyridoxal | Thiosemicarbazide | 10 | 15 | 8 | 12 | 11 | 10 |
| Benzonitrile | Hydroxylamine | 10 | 16 | 9 | 12 | 7 | 10 |
| Menadione | Diphenylsemicarbazide | 10 | 12 | 8 | 15 | 27 | 10 |
| Pyridine-2-aldehyde | Hydrazinobenzothiazole | 17 | M(12) | 8 | 8 | 8 | 10 |
| *p*-Dimethylamino benzaldehyde | Methyl hydrazine | 17 | 9 | 11 | R | 10 | 11 |
| *p*-Dimethylamino benzaldehyde | Benzylphenyl hydrazine | 16 | R | 7 | R | M(10) | 9 |
| Pyridine-2-aldehyde | Phenylethyl hydrazine | 13 | 12 | 18 | R | M(15) | 10 |
| Pyridine-2-aldehyde | Thiosemicarbazide | 9 | M(15) | R | 20 | M(10) | M(9) |
| Methylsalicylate | Hydroxylamine | 9 | R | 9 | 17 | 8 | 11 |
| Ninhydrin | Methylhydrazine | 16 | R | 10 | R | 9 | R |
| 2-Methyl-4-amino-5-chloromethyl pyrimidine | Ethyl-*p*-aminobenzoate | 8 | 16 | M(12) | R | R | M(11) |

*Numbers indicate radial zones of inhibition in mm; R = resistant; M = resistant mutants present, zone of inhibition in parentheses.

not as good as the reaction of *E. coli*. *Staphylococcus aureus* (80/81) responded well only to a few select adducts, especially the menadione analogue. One should like to think that this lack of response is a special attribute of the 80/81 variant, but such data are not yet available. It appears that the bacteria in this study are not too readily suppressed by pyridoxal analogues and that, with the exception of *Pseudomonas aeruginosa*, the menadione derivative holds greater promise against these organisms as a group. Individually, suitable vitamin $B_6$ antagonists can perform quite well in curtailing bacterial proliferation.

TABLE 4. *Carbonyl adducts with good in vitro activity against members of Actinomycetales*

| Carbonyl compound | Carbonyl reagent | *M. tuberculosis* | *N. asteroides* | *N. brasiliensis* |
|---|---|---|---|---|
| Ninhydrin | Hydroxylamine | 11* | 10 | 7 |
| Pyridoxal | Thiosemicarbazide | 10 | 7 | 8 |
| Isatin | Hydrazine | 20 | 11 | 7 |
| Pyridine-4-aldehyde | Thiosemicarbazide | 16 | R | 7 |
| Menadione | Diphenylsemicarbazide | R | 20 | X |
| Salicylaldehyde | Hydrazinoquinoline | R | 11 | 16 |

*Numbers indicate radial zones of inhibition in mm; R = resistant; X = not tested.

Table 4 summarizes the results obtained with the higher bacteria grouped taxonomically. It must be reiterated that the definition of good activity pertains to the growth curtailment of at least one of the organisms under scrutiny, manifested by a zone of inhibition greater than 10 mm. The first four compounds are of interest because they inhibited *M. tuberculosis* (Table 4). Although the ninhydrin and pyridoxal derivatives are not appreciably more efficient in curtailing the growth of *M. tuberculosis* than pyridine-4-aldehyde phenylhydrazone, isatoic hydrazide (the adduct of isatin and hydrazine) and pyridine-4-aldehyde-thiosemicarbazone exerted unquestionably greater inhibitory effects. It is most interesting that the carbonyl reagent thiosemicarbazide appeared twice among the active antitubercular compounds in conjunction with carbonyl compounds closely related chemically. The loss of substitution on the heterocyclic ring of pyridoxal apparently enhances the ability of the adduct to act on *M. tuberculosis*, and substitution of thiosemicarbazide for phenylhydrazine achieves similar improvement. A definite chemical relationship also exists between isatin and ninhydrin. Although pyridine-4-aldehyde thiosemicarbazone displayed no activity against *Nocardia asteroides*, the thiosemicarbazide adduct of pyridine-2-aldehyde inhibited the nocardia quite well (11-mm zone of inhibition). Ninhydrin and pyridoxal adducts each inhibited several of the other microorganisms in varying degrees, isatoic hydrazide displayed only minimal activity against *E. coli* and *B. dermatitidis*, and slight effects were exerted on *E. coli*, *Klebsiella*, *Cryptococcus*, nocardiae,

and *Blastomyces* by pyridine-4-aldehyde thiosemicarbazone. The menadione and salicyaldehyde derivatives were effective against the nocardiae.

The action of these antimicrobial compounds on the yeasts and fungi which cause the systemic mycoses is portrayed in Table 5. Interest in the diseases caused by these parasites cannot be compared with that accorded some of the bacterial infections. This lack of concern perhaps explains the paucity of effective antimycotic agents. However, the reaction of these organisms to the usual chemotherapeutic agents is not very dramatic. The carbonyl adducts found effective in this preliminary study are also not of universal applicability. Certain of the yeasts, especially *C. albicans*, were inhibited by most of the pyridoxal analogues and by the menadione derivative. The proliferation of these fungi, susceptible to menadione-diphenylsemicarbazone, was curtailed quite effectively. The singular response of *Cryptococcus neoformans* to pyrimidine-3-aldehyde-benzylphenylhydrazone is of interest since this compound probably acts as an analogue of nicotinic acid. This yeast reacted also to pyridine-2-aldehyde thiosemicarbazone but the substitution of phenylhydrazine for the thiosemicarbazide caused an appreciable diminution of response. These fungi provided ample opportunity to note the effects of different combinations of carbonyl compounds and carbonyl reagents as well as the substitution of functional groups in various positions of heterocyclic rings. Pyridine-4-aldehyde combined with the same carbonyl reagent increased the susceptibility of the *Cryptococcus* and inhibited *Coccidioides*, the two species of *Blastomyces*, and *Hormodendrum*, all of which were resistant to the adduct formed with the ring substituted in the 3 position. Similar differences became apparent with pyridine-2-aldehyde and quinoline-4-aldehyde when the carbonyl reagents were different. Quinoline-4-aldehyde-phenyl hydrazone warrants special attention. With the systemic fungi, only three, *C. imitis*, *B. dermatitidis*, and *S. schenckii*, were inhibited adequately; however, the dermatophytic fungi responded well to this compound (Table 6). On the other hand, quinoline-2-aldehyde was much less active against the dermatophytes. Pyridine-4-aldehyde benzylphenylhydrazone again showed some superiority to pyridine-3-aldehyde derivatives. It was also generally superior to pyridine-4-aldehyde phenylhydrazone and approximately equal in its activity to pyridine-2-aldehyde thiosemicarbazone with respect to the reaction of the dermatophytic fungi.

Several examples have been presented in the tables displaying action specific against one organism or extraordinary activity against one microbial test object although several of the companion organisms were hardly curtailed. The response of *M. tuberculosis* is typical. One should mention the susceptibility of *C. imitis* to the combination of *p*-dimethyl aminobenzaldehyde and hydroxylamine and of *C. albicans* to pyridoxal and hydrazinophthalylhydrazide as well as pyridine-

TABLE 5. *Carbonyl adducts active against fungi implicated in deep mycoses*

| Carbonyl compound | Carbonyl reagent | Fungi | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | *C. albicans* | *C. neoformans* | *C. immitis* | *B. dermatitidis* | *B. brasiliensis* | *H. pedrosi* | *S. schenkii* | *M. grisea* |
| Menadione | Diphenylsemicarbazide | 23* | 45 | 24 | 19 | 27 | R | R | 25 |
| *p*-Dimethylamino-benzaldehyde | Betaine hydrazide | 18 | R | R | 15 | X | R | 8 | X |
| Pyridine-3-aldehyde | Benzylphenylhydrazine | M(20) | 23 | R | R | R | R | R | X |
| Pyridine-4-aldehyde | Benzylphenylhydrazine | R | 29 | 19 | 33 | 17 | 22 | R | 12 |
| Pyridine-2-aldehyde | Thiosemicarbazide | 13 | 17 | R | 10 | X | 8 | R | X |
| Pyridoxal | *p*-Nitrophenylhydrazine | 15 | R | 20 | 7 | X | R | R | X |
| Quinoline-4-aldehyde | Semicarbazide | 12 | R | 16 | 7 | X | R | 14 | X |
| Diethylaminoacetonitrile | Hydroxylamine | 12 | 8 | 16 | 10 | X | R | 8 | X |
| Pyridoxal | Semicarbazide | 12 | 8 | 20 | 10 | X | R | R | X |
| Pyridine-2-aldehyde | Phenylhydrazine | 9 | 10 | 20 | 11 | X | 21 | R | X |
| Benzonitrile | Hydroxylamine | 13 | R | 20 | 13 | X | R | R | X |
| Isonicotinic ester | Hydroxylamine | 13 | R | R | 14 | X | 18 | 8 | X |
| Veratraldehyde | Hydrazine | 11 | 10 | R | 12 | X | 28 | R | X |
| Quinoline-4-aldehyde | Phenylhydrazine | 9 | 9 | 14 | 10 | X | R | 16 | X |

*Numbers indicate radial zones of inhibition in mm; R = resistant; M = mutants present, zone of inhibition in parentheses; X = not tested.

TABLE 6. *Carbonyl adducts active against dermatophytic fungi*

| Carbonyl compound | Carbonyl reagent | Fungi | | | |
|---|---|---|---|---|---|
| | | *M. gypseum* | *M. canis* | *M. audouini* | *T. mentagrophytes* |
| Quinoline-4-aldehyde | Phenyl hydrazine | 16* | 20 | 28 | 12 |
| Menadione | Diphenyl semicarbazide | 16 | M(17) | 39 | M(26) |
| Quinoline-2-aldehyde | Diphenyl semicarbazide | 17 | R | R | R |
| Ninhydrin | Benzyl phenylhydrazine | 16 | M(12) | R | R |
| Pyridine-3-aldehyde | Benzyl phenylhydrazine | 15 | M(8) | 25 | M(19) |
| Pyridine-4-aldehyde | Benzyl phenylhydrazine | 36 | 22 | 20 | 8 |
| Pyridine-4-aldehyde | Phenyl hydrazine | 23 | 11 | 30 | 12 |
| Pyridine-2-aldehyde | Thiosemicarbazide | 22 | 21 | 20 | 14 |

*Numbers indicate radial zones of inhibition in mm; R = resistant; M = mutants present, zone of inhibition in parentheses.

2-aldehyde combined with *p*-sulfophenylhydrazine. In this same category are the response of *S. aureus* (80/81) to alloxan and diphenylsemicarbazide and the action of *p*-dimethylaminobenzaldehyde combined with benzylphenylhydrazine on *E. coli*, the pyrimidal moiety of thiamine and *p*-amino benzoate and pyrimidine and ethyl *p*-aminobenzoate on *Klebsiella*, although the same thiamine fragment joined to pyrimidine-nicotinonitrile inhibited *P. aeurginosa*. One wonders whether from these many different reactions to the 192 combinations one can form a pattern which would delineate certain functional groups more effective in combination against microorganisms. Since the frequency with which the different carbonyl compounds and carbonyl reagents were employed differed, a comparison of their successful action was possible only in terms of the frequency with which they were effective as compared to the frequency with which they occurred in the adducts. The results were restricted further by limiting inclusion as an active compound to those combinations which resulted in inhibition zones greater than 10 mm (Table 7). A limited number of the compounds were not tested against some of the organisms but this was ignored for purposes of comparison. Consideration of the percentage of activity indicates that phenylhydrazine and diphenylsemicarbazide are the most promising of the carbonyl reagents, and menadione stands out among the carbonyl compounds (Table 7). One must, however, stress the striking specific activity of certain of the compounds such as isatin which would be neglected by this interpretation. Table 7 certainly suggests that some combinations not reported here might prove worthwhile. Such additional variations are now under investigation.

TABLE 7. *Frequency with which constituents occurred in effective antimicrobial adducts*

| Reagent* | A<br>Number of times effective (10 mm or more) against 21 microorganisms | B<br>Number of times in combination to form adducts | Activity $\left(\frac{A}{B \times 21} \times 100\right)$ |
|---|---|---|---|
| | | | % |
| Diphenylsemicarbazide | 13 | 9 | 6.9 |
| Benzylphenylhydrazine | 12 | 17 | 3.4 |
| Thiosemicarbazide | 10 | 20 | 2.4 |
| Phenylhydrazine | 9 | 6 | 7.1 |
| Hydroxylamine | 7 | 13 | 2.5 |
| Methylhydrazine | 4 | 13 | 1.4 |
| Semicarbazide | 3 | 5 | 2.9 |
| Menadione | 12 | 3 | 19.1 |
| Pyridine-4-aldehyde | 23 | 17 | 6.4 |
| Pyridine-2-aldehyde | 10 | 15 | 3.1 |
| Quinoline-4-aldehyde | 8 | 7 | 5.4 |
| *p*-Dimethylaminobenzaldehyde | 5 | 10 | 2.3 |
| Pyridoxal | 4 | 8 | 2.3 |
| Ninhydrin | 3 | 10 | 1.4 |

*The activity of other constituents ranged from 0.5 to 2.3.

## Literature Cited

Albert, A. 1960. Selective toxicity. John Wiley and Sons, Inc., New York.

Finland, M. 1960. Treatment of pneumonia and other serious infections. New Engl. J. Med. **263**: 207-221.

Grove, D. C., and W. A. Randall. 1955. Assay methods of antibiotics. Medical Encyclopedia, Inc., New York.

Isenberg, H. D., and S. Shapiro. 1958. An inexpensive, easily constructed microbiological zone reader. Antibiotics & Chemotherapy **8**:466-469.

Seifter, E., E. Henson, and H. D. Isenberg. 1962. A series of synthetic antimicrobial compounds. I. Chemistry. Antimicrobial Agents and Chemotherapy - 1961, p. 823-829.

Woodruff, H. B., and L. E. McDaniel. 1958. The antibiotic approach. Symposium Soc. Gen. Microbiol. **8**:29-48.

Woods, D. D., and R. G. Tucker. 1958. The relation of strategy to tactics: some general biochemical principles. Symposium Soc. Gen. Microbiol. **8**:1-28.

Woolley, D. W. 1958. The designing of antimetabolites. Symposium Soc. Gen. Microbiol. **8**:139-162.

# CONTROLLED TESTS OF DISINFECTANTS UNDER "IN-USE" CONDITIONS

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In recent years the increasing number of hospital-acquired staphylococcal infections has caused a revival of interest in methods designed to control the spread of bacteria within the hospital environment (Koch et al., 1959; Mudd, 1958). Many references are available implicating various factors involved with this resurgence of bacterial infection within our communities and hospitals (Nahmias and Eickhoff, 1961), including contact with hospital personnel carrying these organisms (Vogel et al., 1959; Wise et al., 1959), poor architectural design (Hurst et al., 1958; Nahmias and Eickhoff, 1961), the widespread use of both prophylactic and therapeutic antibiotics (Klarmann, 1959), inadequate training of hospital personnel (e.g., Adams et al., 1959; Adams and Fahlman, 1960), poor housekeeping procedures (e.g., Colbeck, 1960; Howe et al., 1961; McLean, 1961; Walter and Kundsin, 1960), and the improper use of nursing and isolation techniques (e.g., Dineen, 1960; Goodall, 1952). In certain hospitals the infection rate has reached alarming proportions, and drastic measures have been necessary before these infections were partially or totally controlled (Dineen, 1960; Wysham and Kirby, 1957).

Any effective method used for environmental disinfection must be based upon a rational use of an effective germicidal compound. These compounds should: have an effective initial and residual germicidal activity, be nontoxic to hospital personnel and patients, not injure the surfaces to which they are applied, be easily applied, have no noxious colors or odor, and be relatively inexpensive.

At the Medical College of Virginia Hospital, Richmond, Va., the effect of various disinfectant solutions upon the bacteria commonly found within the hospital environment was studied after the construction of a pathogen-free environment designed for long-term patient care. In attempting to evaluate the best disinfectant to use within this highly specialized area, a mass of conflicting reports were encountered concerning the effectiveness of various agents. It was impossible on the basis of the data gleaned from the literature to make a rational decision as to the agent of choice. It was then

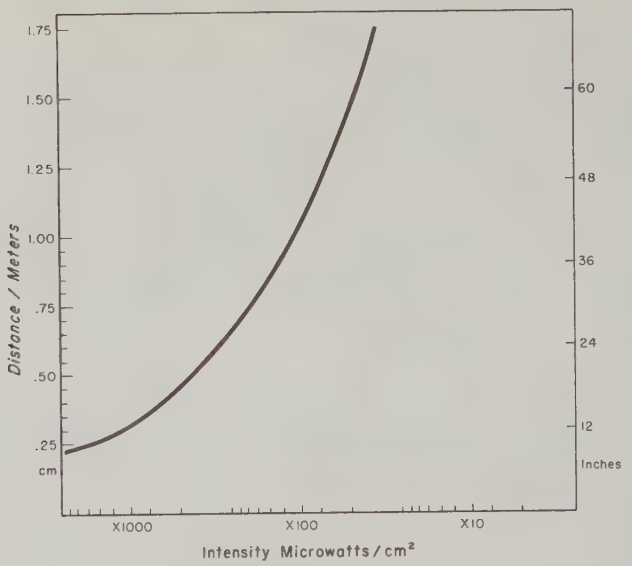
decided that a modified "in-use" testing procedure should be set up to carry out the necessary evaluation of these agents.

The methods used most often for evaluating the disinfectant activity of various solutions (A.O.A.C. phenol coefficient method, the "use-dilution" method of Stuart et al.) and other laboratory testing procedures leave much to be desired (Klarmann, 1959, 1960; Walter and Kundsin, 1960). The main disadvantage of these methods is that there is little relationship between the laboratory methods used in these tests and the actual environment in which the disinfectant solutions must be applied. The effects of temperature, humidity, reinoculation of bacteria onto the surface, the damage to the surface resulting from the application of the germicidal reagent, and the variation of the types of bacterial contamination cannot be adequately reproduced with these methods. Therefore, an attempt has been made to set up a testing procedure by which controlled concentrations of bacteria can be applied to surfaces identical to those found within the hospital area, and that would allow the sequential evaluation of the other variables. It is realized that no laboratory testing procedure can completely duplicate the conditions found within a busy hospital ward.

The present report is concerned with the methodology evolved to carry out these tests, the initial results obtained in relation to the bacterial strains and disinfectant agents used, and the type of surface being disinfected. The associated effects of temperature and humidity variations, repetitive reinoculation of the test surfaces, the use of mixed flora, and surface contamination with other materials commonly found within the hospital environment have not as yet been studied.

## Materials and Methods

A closed laboratory hood was used as the test site. A sliding glass panel enclosed the front of the hood, and a ventilation fan evacuated any bacteria that had not settled to the test surface. This hood was modified as follows: (i) A one-way damper was placed on the ventilation outlet so that there would be no reflux of air to disturb the fall-out patterns of bacteria sprayed into the hood. (ii) A hole (1 cm diam) was bored through the side of the hood $2\frac{1}{4}$ feet above the test surfaces, so that the bacteria could be sprayed into the closed space. (iii) A variable-intensity ultraviolet light was placed to allow the amount of radiation used to be carefully controlled (calibrated from the data in Fig. 1). (iv) A mercury manometer calibrated to 1,500 mm of mercury was placed on the site of the hood and connected to a spray apparatus consisting of an atomizer, nebulizer, and a pressure pump. These changes are shown in Fig. 2.



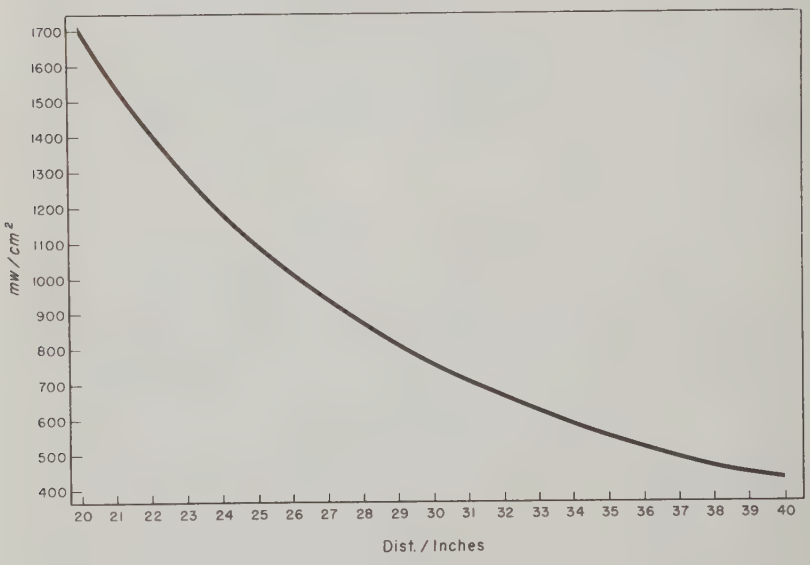




FIG. 1. *Ultraviolet light intensity (top). Semi-log plot for ultraviolet tube (bottom). Same data for use in hood. Readings made with a Westinghouse UV Indicator Meter for Steri-Lamps.*

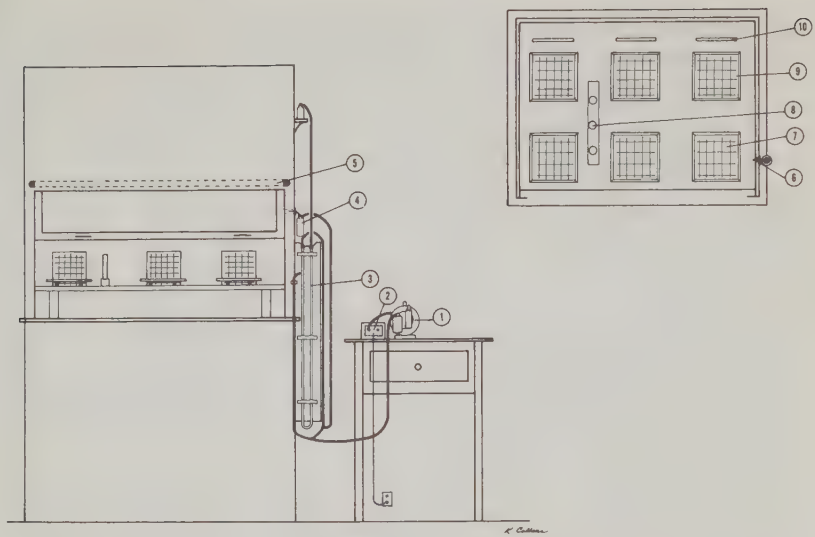



FIG. 2. *Diagram of hood. 1, pressure pump; 2, timer; 3, mercury manometer; 4, nebulizer; 5, ultraviolet light; 6, opening for spray; 7, floor model; 8, stainless-steel model; 9, bedside-table model; 10, wall model.*

*Selection of bacteria.* The selection of the types of bacteria used for these studies was determined by the frequency with which these organisms were recovered from the hospital environment (Table 1).

TABLE 1. *Frequency of recovery of bacteria from a hospital environment**

| Organism | Surgical ward | | Isolation ward | | Operating room | | Anesthesia | | Total | |
|---|---|---|---|---|---|---|---|---|---|---|
| | No. | Per cent | No. | Per cent | No. | Per cent | No. | Per cent | No. | Per cent |
| *Staphylococcus* (coagulase-neg.) | 241 | 19.3 | 15 | 14.1 | 895 | 49.3 | 370 | 57.4 | 1521 | 39.9 |
| Coliform | 256 | 20.6 | 17 | 16.1 | 25 | 1.5 | 5 | 0.8 | 303 | 7.9 |
| *Pseudomonas* | 48 | 3.8 | 5 | 4.7 | 14 | 0.8 | 72 | 11.2 | 139 | 3.6 |
| *Proteus* | 5 | 0.4 | 2 | 1.9 | 2 | 0.1 | 0 | – | 9 | 0.2 |
| *Staphylococcus* (coagulase-pos.) | 82 | 6.6 | 13 | 12.3 | 12 | 0.7 | 10 | 1.5 | 117 | 3.1 |

*Number of cultures taken: surgical ward, 1,244; isolation ward, 106; operating room suite, 1,818; anesthesia room, 645. Total: 3,818.

A total of 3,818 cultures were taken. All cultures were taken with swabs moistened with thioglycolate broth, incubated for 18 to 24 hr, and then subcultured on 5% blood agar plates and other media necessary for identification.

The most frequent organism recovered was *Staphylococcus albus* (coagulase negative). The next most frequent group of organisms was

the coliform group, of which *Escherichia coli* is the major member. This organism was most often obtained from cultures of the wards, the hall, and ward floors. *Pseudomonas* was recovered in 3.6% of all of the cultures, with approximately 56% of all cultures taken from the anesthetic corrugated tubing showing this organism. *S. aureus* (coagulase positive) was found on 3.1% of all of the swabs taken, with the highest number of positive cultures being found outside of the isolation units and within the hospital wards. This organism is not often recovered within the operating room suites. *Proteus mirabilis* was found very infrequently, but it is included in the strains being studied as another representative of the coliform group of bacteria. Thus, the coagulase-negative staphylococci, *E. coli*, and *Proteus* organisms are among those most frequently recovered from the environmental cultures, and have been used as the test organisms in the present testing procedure. Some difficulties have been encountered with *Pseudomonas* strains, and a more intensive study of this organism is now underway.

*Standard curve construction.* Standard curves have been constructed by relating the concentration of solutions of coagulase-negative staphylococci, *E. coli*, and *P. mirabilis* to the per cent light transmission of the solutions at 650 m$\mu$ in a Coleman Junior spectrophotometer.

The organisms were grown on a brain veal agar slant for 18 hr at 37 C. A second slant was inoculated with sterile 5% dextrose and water, to serve as a blank, and was also incubated for 18 hr.

The bacterial growth was then washed from the slant, using 5 ml of 5% dextrose and water and glass beads. This solution was then diluted six times (to $\frac{1}{64}$ of its original concentration), using 5% dextrose and water.

The per cent of light transmitted by the solution at a wave length of 650 m$\mu$ was determined for each of the dilutions on a Coleman Junior spectrophotometer.

A colony count was made on each sample, after multiple dilutions to $1 \times 10^{9}$. The final dilution was placed on a 5% blood agar plate and incubated for 18 to 24 hr. The colonies were then counted, and the concentration of viable bacteria per ml of the original solution determined.

Satisfactory curves for pseudomonads were obtained after adding 0.3 ml of 0.2% Versenate to 5 ml of the sample, to prevent clumping. It was impossible to obtain reproducible values for light transmission until this was done.

With *P. mirabilis*, 0.1 ml of the final dilution was added to liquid agar, which was then poured onto a 5% blood agar plate. This prevents excessive spreading of this organism and allows adequate colony counts to be obtained.

The per cent transmission and the number of bacteria per ml were then plotted on a standard semi-log graph (Figs. 3-5). The

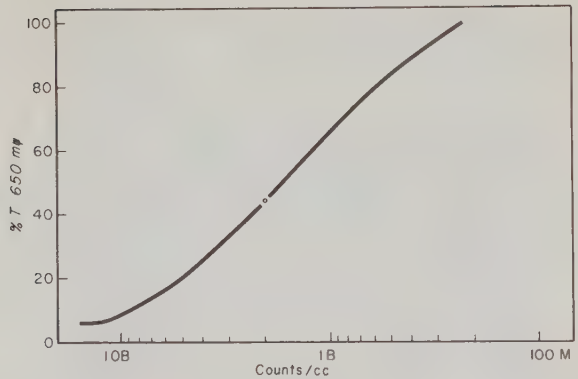



FIG. 3. *Staphylococcus (coagulase-negative) standard curve; 42% transmittance (T) equivalent to 2 billion organisms.*

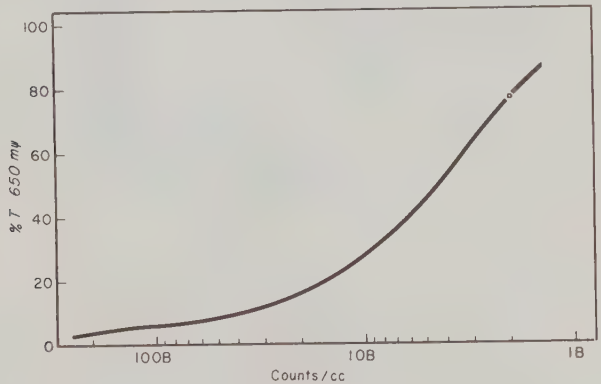



FIG. 4. *Escherichia coli standard curve; 73% T equilivant to 2 billion organisms.*

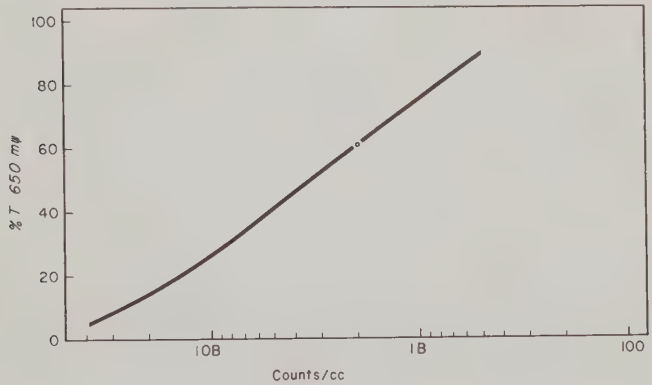



FIG. 5. *Proteus mirabilis standard curve; 62% T equilivant to 2 billion organisms.*

values were rechecked at frequent intervals to insure reproducibility.

## Results

A total of 21 determinations were made for coagulase-negative staphylococci, but it was necessary to delete 4 of these from the calculations, owing to technical errors. The mean colony count (in billions) was 2.13, with a standard deviation of $\pm 0.34$. Nine determinations of *E. coli* at 73% transmission yielded a mean count of $2.24 \pm 0.28$ billion organisms per ml. Only six determinations for *P. mirabilis* were performed, using the standard curve and reading at 52% transmission; these showed a mean count of $2.18 \pm 0.19$ billion organisms per ml. These results were all obtained routinely during the testing procedure.

It is very necessary to use fresh solutions of bacteria. Table 2 shows the effect of storage at -12 C for various periods of time on a strain of coagulase-negative staphylococci. It is apparent that under these conditions nonviable bacteria remain in the solution and give falsely high counts. The per cent error increased with time.

TABLE 2. *Effect of refrigeration on a strain of coagulase-negative staphylococci*

| Time | Transmittance | Expected count (std. curve) | Measured count* | Error |
|---|---|---|---|---|
| *hr* | % | | | % |
| 0 | 46 | 2 Billion | 1.83 B | 9.3 |
| 24 | 60 | 1.25 Billion | .56 B | 55 |
| 48 | 65 | .91 Billion | .61 B | 33 |
| 120 | 68 | .8 Billion | .09 B | 89 |

*Counted at the same time transmittance was determined.

*Application of bacteria to the test surfaces.* By use of standard curves, known concentrations of bacteria were applied to the test surface by swabbing or by spraying through an atomizer or nebulizer (Fig. 6). The spray pattern obtained depends upon the type of applicator used (atomizer or nebulizer), the pressure at which the bacteria are sprayed, and the time of spraying.

The organism most frequently used in these studies has been a coagulase-negative staphylococcus. The initial applications were made using a nebulizer that caused large droplets to be formed. Various pressure and time sequences were tested, but the spray pattern obtained was excessively heavy and the exposed plates could not be counted accurately, since the colonies were frequently superimposed upon each other. Accordingly, a deVilbiss nebulizer was used

FIG. 6. *Atomizer (left) and nebulizer (right) used to spray organisms.*

that gave a much finer spray pattern. The nebulizer spray pattern was first studied by placing large agar-covered glass plates on the fall-out area and then performing multiple colony counts on different sections of the plates after incubation at 37 C. The average number of colonies was shown to be $12.8/cm^3$ ($\pm 0.12$). Later, 5% blood agar plates were used to sample the bacterial fall-out pattern, by exposing the plates next to the test surfaces while the bacteria were being sprayed.

The pressure with which the organisms are sprayed is of prime importance in determining the spray pattern obtained. Table 3 summarizes the spray pattern resulting when the same dilutions of organisms are sprayed, using varying pressures. The percentage of

TABLE 3. *Effect of pressure on nebulizer spray pattern of staphylococci*

| Pressure | Average colony count/$cm^2$ | Average error |
|---|---|---|
| *mm/Hg* | | % |
| 600 | 79 | ± 23.1 |
| 760 | 109 | ± 10.5 |
| 1040 | 10.5 | ± 60.1 |

error is less using a pressure of 760 mm of mercury. The average colony count varies only ±10.5%. When excessively high pressures are used, the number of viable bacteria falling onto the test surface is markedly decreased but the error markedly increases.

The time of application of the spray can be varied, but for convenience 1 min was used for all of the tests. The average colony count per $cm^2$ was 75 ± 27, using coagulase-negative staphylococci.

Useful spray patterns have not been obtained for *E. coli* with this method. Therefore, *E. coli* was initially wiped onto the test surfaces in a concentration of approximately 2 billion organisms per ml. After the initial cleaning of the surface with the disinfectant being tested, the bacteria were then sprayed onto the test surface with the atomizer. This gave an evenly distributed growth pattern too heavy to be accurately counted.

*Proteus* and *Pseudomonas* were applied in a heavy spray during all phases of the tests. The atomizer was used for *Proteus*, and the nebulizer for *Pseudomonas*.

*Test surfaces.* The test surfaces are analogues of the hospital surfaces (floors, walls, tops of bedside tables, and stainless steel piping). The floor models are covered with Armstrong Carbon Vinyl plastic and the bedside table models are covered with Formica laminated plastic tops. The pipe used is stainless steel, and the wall model is made of plaster painted with Sampson paint. The floor and bedside table models are flat during the test procedure and the pipe stands upright. The wall models are arranged vertically so that the fall-out is similar to that occurring in the hospital ward.

*Test procedure.* Prior to the test, the entire inner surface of the hood and the test surfaces are cleaned with soap and water and then exposed to intense ultraviolet light for 10 min. The amount of radiation used is sufficient to sterilize these surfaces and does not alter the test surfaces for these purposes. One-half of each surface is used as the test area and the other as a control.

A known concentration of one of the organisms is obtained by use of the standard curve, and this is applied to the test surface. The organisms are allowed to settle onto the test area for 10 min after the initial spray application. Then the exhaust fan is turned on for 1 to 2 min. One-half of each surface area is then cleaned with the disinfectant solution being tested, while the uncleaned surface is used as a control area. When direct application is used, the disinfectant is applied by using sterile gauze and gloves. After drying, the initial cultures are taken from both the test and control areas, using swabs moistened with thioglycolate broth. When the organisms are directly applied, they are allowed to dry for 10 min before the disinfectant is applied. After these initial cultures have been taken, the bacteria are again sprayed onto the test surfaces and repeat cultures are taken immediately. Subsequent cultures are taken at various intervals. The cultures taken after the cleaning of

the bacteria from the surface with the disinfectant tests both the initial cleaning and the bactericidal activity of the compound, and the immediate residual activity is determined by the cultures taken after the reapplication of the bacteria. Further residual bactericidal effects are determined by taking repetitive cultures of both the test and control areas at various time intervals. Positive cultures must be obtained at the same time from the control area before any importance is attached to a negative culture taken from the adjacent test area.

The swab samples are incubated for 18 hr at 37 C in the thioglycolate broth. At the end of this time, they are read as "positive" or "negative." Cultures of *Pseudomonas, Proteus,* and *E. coli* have consistently been readable within this period, but cultures of *Staphylococcus* are often not readable within this time limit; all negative broth cultures are subcultured onto a 5% blood agar plate.

TABLE 4. *Initial results with full strength disinfectants*

| Disinfectant | Organism | Positive cultures (%) | | | | | |
|---|---|---|---|---|---|---|---|
| | | Clean | Respray | 1 Hr | 2 Hr | 4 Hr | Total |
| Tergisyl | *S. aureus* | 0 | 50 | 0 | 0 | 0 | 9 |
| | *E. coli* | 0 | 0 | 0 | 12.5 | 12.5 | 5 |
| Amphyl | *S. aureus* | 10 | 50 | 11 | 0 | 0 | 15 |
| | *E. coli* | 12.5 | 25 | 12.5 | 25 | 12.5 | 18 |
| Sandril | *S. aureus* | 37.5 | 62.5 | 0 | 0 | 14 | 24 |
| | *E. coli* | 0 | 50 | 0 | 0 | 0 | 10 |
| Staphene | *S. aureus* | 0 | 71 | 15 | 0 | 17 | 22 |
| | *E. coli* | 12.5 | 75 | 12.5 | 12.5 | 25 | 28 |
| Permachem | *E. coli* | 25 | 62.5 | 25 | 31.3 | 18.6 | 34 |
| SBT-24 | *S. aureus* | 0 | 80 | 50 | 12.5 | 17 | 34 |
| | *E. coli* | 12.5 | 75 | 75 | 37.5 | 25 | 45 |
| Wescodyne | *S. aureus* | 0 | 70 | 60 | 50 | 67 | 40 |
| Dicrobe | *S. aureus* | 0 | 100 | 17 | 0 | 17 | 27 |
| | *E. coli* | 37.5 | 100 | 75 | 62.5 | 50 | 65 |
| Betadyne | *S. aureus* | 14 | 93 | 43 | 43 | 80 | 52 |
| | *E. coli* | 37.5 | 100 | 100 | 37.5 | 62.5 | 68 |
| Warexin | *S. aureus* | 0 | 80 | 67 | 87.5 | 86 | 64 |

After the techniques had been standardized, the compounds listed in Table 4 were studied. The active ingredients in each compound are listed in Table 5. In the initial test procedures, all of the compounds were applied to the test surfaces in the undiluted solution supplied by the manufacturer. The immediate and the residual bactericidal activity after 4 hr was determined.

The results of these initial studies are summarized in Table 5. Each compound had a minimum of 8 cultures taken during each culture period for each organism studied, and several of the compounds had 16 cultures taken for each test period. The results are tabulated to show the percentage of positive cultures obtained for each organism

with each disinfectant. As in all the test procedures, the surfaces were divided into control and test areas. The percentage of positive cultures was determined by dividing by the number of positive cultures obtained from the control area.

TABLE 5. *Active ingredients in the disinfectants used in this study*

| Disinfectant | Ingredients |
|---|---|
| S.B.T.-24 (Lever Brothers) | *n*-Propyl alcohol, 3,4,5-cribromosalicylanilate, 4,5-dibromosalicylanilide, lauryldecethylenetriamine, ethylenediaminetetracetic acid |
| Tergisyl (Lehn & Fink) | Sodium xylene sulfonate, triethanolamine dodecyl benzene sulfonate, *o*-hydroxydiphenyl, *p*-tertiary amylphenol |
| Amphyl (Lehn & Fink) | Potassium ricinolate, *o*-phenylphenol, *p*-tertiary amylphenol, alcohol |
| Permachem (Permachem, Inc.) | Dialkyl dimethyl ammonium chloride, bis-(tri-*n*-butyltin) oxide, salicylic acid, isopropanol |
| Sandril (Taylor Hales Laboratories) | Isopropanol, *o*-benzyl-*p*-chlorophenol, *o*-chloro-*o*-phenylphenol, *p*-chloro-*o*-phenylphenol, tetrasodium ethylenediaminetetraacetate |
| Staphene (Vestal, Inc.) | Soap, *o*-benzyl-*p*-chlorophenol, *p*-tert-amylphenol, isopropanol, *o*-phenylphenol, tetra-sodium ethylenediaminetetraacetate (active ingredients, 30.8%). |
| Dicrobe (Huntington Laboratories) | Isopropanol, disodium 4-dodecyl-2,4-oxydibenzenesulfonate, sodium *o*-benzyl-*p*-chlorophenate, sodium deodecyl benzenesulfonate, and disodium 2,2-oxybis (4-dodecyl benzenesulfonate) |
| Warexin (Davol Rubber Co., Guardian Chemical Corp.) | Tetradecylbenzenesulfonate, hypochlorous acid complex |
| Wescodyne (West Chemical Products, Inc.) | Polyethoxy polypropexy polyethoxy ethonoliodine complex, nonyl-phenoxy polyethoxy ethonol-iodine complex, hydrochloric acid |
| Betadyne (Talby Nason Co.) | Polyvinyl-pyrrolidone (povidove)-iodine complex |

Coagulase-negative staphylococci were used for the initial testing procedure. After this series of tests had been completed, *E. coli* was substituted as the test organism, and the tests were repeated. Warexin and Wescodyne were tested only against the staphylococci; the results did not warrant further tests. Warexin is primarily advocated for instrument sterilization (Engelhard, Wieldman, and Jolliff, 1961). The Permachem solution was tested only with *E. coli;* this compound was not available during the initial testing with staphylococci.

The lowest number of positive cultures was obtained with Tergisyl. Against *E. coli,* only 2 of 40 cultures were positive during the test period; with coagulase-negative staphylococci, the only positive cultures were obtained immediately after respraying. Amphyl, containing similar active ingredients, showed essentially the same culture pattern, although a few more positive cultures were obtained. Sandril was especially effective against *E. coli,* although the effect

upon staphylococci was not as rapid. The results obtained with Staphene were essentially similar for both organisms. SBT-24 eliminated both organisms from the surfaces, but residual activity appeared weak. Dicrobe was effective against staphylococci but relatively ineffective against *E. coli*. Betadyne exhibited a low degree of activity against both organisms. Betadyne was developed primarily for application to the skin, but it has been advocated for environmental disinfection; it is an iodophor similar to Wescodyne (Klarmann, 1960).

The greatest activity was shown by Tergisyl, Amphyl, Sandril, and Staphene. All of these are basically phenolic substituted compounds. Amphyl is very similar to Tergisyl but has no detergent effect, and further tests were not carried out with this compound. Therefore, Tergisyl, Sandril, and Staphene were chosen for further testing procedures.

After the initial screening procedures, Tergisyl, Sandril, and Staphene were tested in various concentrations against coagulase-negative staphylococci and *E. coli*. The results of these test procedures are shown in Table 6. The "use" dilution refers to that dilution recommended by the manufacturer. The 4, 10, and 25% solutions were made from the stock solution obtained from the manufacturer. The results with the "use" and 4% dilutions are very disappointing. A 10% solution is more effective, and even better results are obtained with the 25% solutions. The best results were obtained with Tergisyl; there was essentially little difference in the results obtained with Sandril and Staphene. Both of these compounds were somewhat more active against staphylococci. Because of the marked improvement in effectiveness with the 25% solution, this strength of each compound was used for the other test procedures.

TABLE 6. *Effect of disinfectant concentration*

| | | Per cent concn | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | Use | | 4 | | 10 | | 25 | |
| Disinfectant | Organism | No. | Per cent | No. | Per cent | No. | Per cent | No. | Per cent |
| Tergisyl | *S. aureus* | 30 | 80 | 59 | 66 | 23 | 30 | 32 | 9 |
| | *E. coli* | 36 | 92 | 28 | 93 | 32 | 41 | 40 | 5 |
| | Average | 66 | 86 | 89 | 75 | 55 | 36 | 72 | 6.9 |
| Sandril | *S. aureus* | 32 | 69 | 57 | 49 | 30 | 23 | 38 | 24 |
| | *E. coli* | 36 | 97 | 28 | 100 | 32 | 56 | 40 | 10 |
| | Average | 68 | 84 | 85 | 66 | 62 | 40 | 78 | 16.7 |
| Staphene | *S. aureus* | 34 | 79 | 51 | 43 | 26 | 31 | 65 | 22 |
| | *E. coli* | 36 | 94 | 28 | 89 | 32 | 44 | 40 | 28 |
| | Average | 70 | 87 | 79 | 59 | 58 | 38 | 105 | 23.8 |

The germicidal effects of Tergisyl, Sandril, and Staphene are summarized in Fig. 7. With staphylococci, 190 positive control cultures were obtained for Tergisyl, 181 for Sandril, and 184 for Staphene.

The numbers of cultures obtained for each culture period are similar. All of the disinfects initially cleaned the bacteria from the test surfaces very well. Staphene killed fewer bacteria within the immediate culture period after the respraying of the organisms than did Tergisyl or Sandril. After this period, Tergisyl gave the lowest number of positive cultures. Over-all, there is very little difference between these disinfectants.

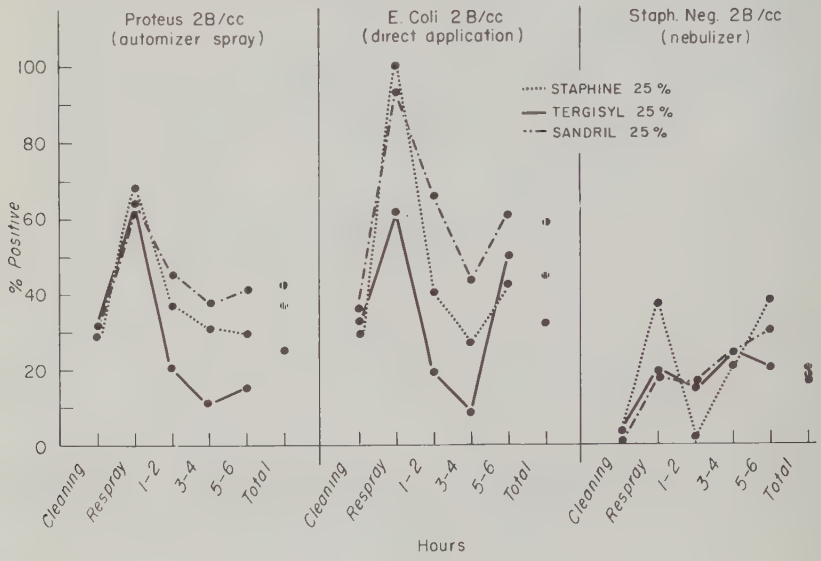



FIG. 7. *Effect of disinfectants against test organisms.*

When *E. coli* was used as the test organism, the number of positive controls obtained for each disinfectant was 192. Again, all of the disinfectants initially showed approximately equal clearing of test surfaces, but Tergisyl showed more rapid activity after reapplication of the organism. The residual effect of all of the disinfectants was decreased after 3 to 4 hr; Tergisyl showed 8% of the cultures to be positive, Staphene 27%, and Sandril 43%. Although the percentage of positive cultures obtained was higher with *E. coli*, Tergisyl again gave fewer positive cultures than the other two disinfectants.

When *P. mirabilis* was used as the test organism, there was very little difference in the percentages of positive cultures obtained, in comparison with *E. coli*, but activity persisted longer. There was no significant difference between Sandril and Staphene during any culture period.

Figure 8 shows the effect of each disinfectant upon the individual organisms. Tergisyl is most effective against staphylococci but is

less active against the gram-negative organisms. The only difference between the effects upon *P. mirabilis* and *E. coli* is shown at 5 to 6 hr, when the residual activity against *E. coli* has decreased fairly markedly.

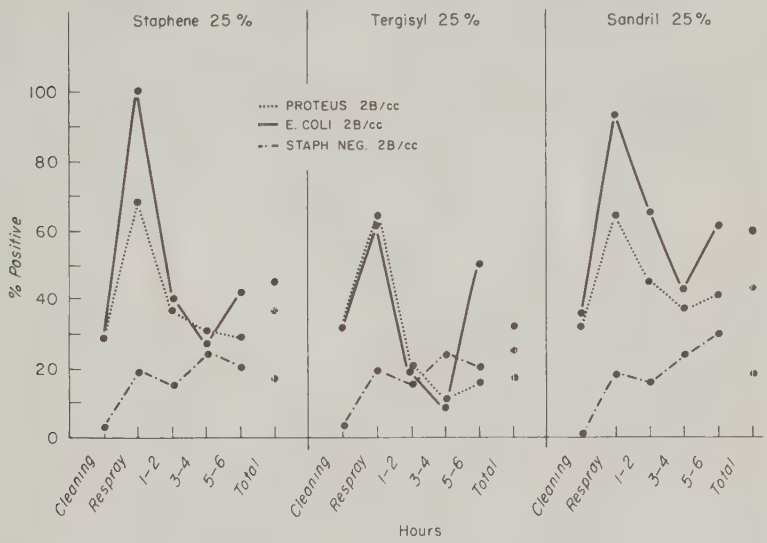



FIG. 8. *Effect of Staphene, Tergisyl, and Sandril against test bacteria.*

With Sandril, again the staphylococci are most sensitive, followed by *P. mirabilis* and then *E. coli*.

The general effect with Staphene is similar, but over-all Staphene is slightly more effective than Sandril. Again *E. coli* is the most resistant of the strains tested, and the staphylococcus is the most sensitive.

Figure 9 shows the effect of each disinfectant upon all of the strains tested. With Tergisyl, 573 positive cultures were obtained over the test period, with Sandril 555, and with Staphene 567. Staphene gave a slightly lower number of positive controls after cleaning. After reapplication of the bacteria, Tergisyl showed the greatest activity, followed by Sandril and Staphene. The residual activity for the subsequent culture periods showed a similar trend, in that Tergisyl gave the lowest number of positive cultures, followed by Staphene and then Sandril. The total effect of the disinfectant during the test period was calculated: 25% of the cultures were positive using Tergisyl, 34% using Staphene, and 40% using Sandril.

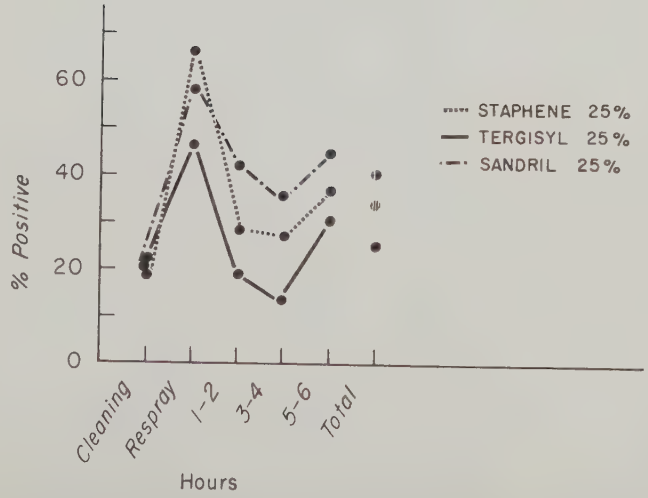



FIG. 9. *Summary of the disinfectant action on Proteus, E. coli, and S. aureus.*

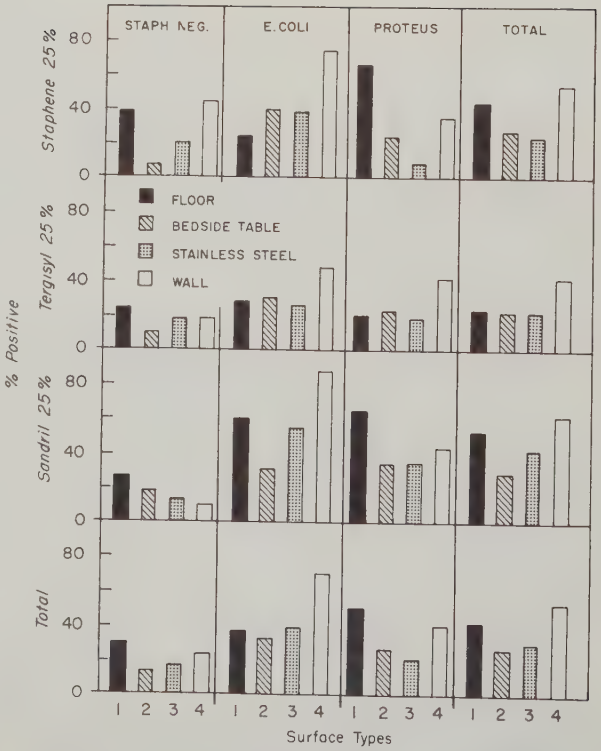



FIG. 10. *Effect of disinfectants on various test surfaces.*

Figure 10 relates the results to the material used as a test surface. For all of the organisms studied, more positive cultures were obtained from the wall and floor surfaces than from the surface of the bedside tables and the stainless steel piping. When the data are summarized according to the type of disinfectant used, the floor and wall surfaces again consistently give a higher number of positive cultures. In summary, 53% of 322 positive control cultures from wall surfaces and 41% of 385 positive control cultures from floor surfaces were positive; only 26% of 386 positive control cultures from the bedside table, and 30% of 308 positive control cultures from the stainless steel piping were positive. There are minor variations within each grouping but these are not significant.

## Discussion

There are several problems concerning the testing procedure itself that must still be evaluated, but the conditions can be made more similar to those found within a hospital environment. The degree of contamination used in each test procedure is usually larger than that generally found upon hospital surfaces, but even greater concentrations of bacteria have occasionally been reported.

Under the conditions of this test, none of the disinfectants consistently and completely eradicates the bacteria. This study comprises only a small section of that being undertaken of the entire pattern of environmental bacteriology found within one hospital. Manifold efforts must be made by the medical, nursing, and administrative staff to effect a decrease in the bacteriological population of any hospital environment. The use of effective disinfectant solutions is a major part of any bacteriological control program, but it must be stressed that the correct application of isolation, nursing, and cleaning techniques is of prime importance. Probably the most important facet of any bacteriological control program is the education to and awareness of the problem of bacteriological spread on the part of the personnel concerned.

## Acknowledgments

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The disinfectants used were generously supplied by the manufacturers.

## Literature Cited

Adams, R., B. Fahlman, E. W. Dube, F. J. C. Dube, and S. Read. 1959. Control of infections within hospitals. J. Am. Med. Assoc. **169**:1557-1567.

Adams, R., and B. Fahlman. 1960. Sterility in operating rooms. Surg. Gynecol. Obstet. p. 367-376.

Colbeck, J. C. 1960. Environmental aspects of staphylococcal infections acquired in hospitals. Am. J. Public Health **50**:468-473.

Dineen, P. 1960. A suggested method for the control of staphylococcal infections in a large general hospital. Am. J. Surg. **100**:534-538.

Engelhard, W. E., J. G. Wieldman, and C. R. Jolliff. 1961. Evaluation of Warexin as a potential cold sterilizing agent. Surgery **49**:651-656.

Howe, C. W., T. F. Silva, Jr., A. T. Marston, and D. D. B. Woo. 1961. Staphylococcal contamination of mattresses and blankets on a surgical ward under nonepidemic conditions. New Engl. J. Med. **264**:625-632.

Hurst, V., M. Grossman, F. R. Ingram, and A. E. Lowe. 1958. Hospital laundry and refuse chutes as source of staphylococcic cross-infection J. Am. Med. Assoc. **167**:1223-1228.

Goodall, J. W. D. 1952. Cross-infection in hospital wards; its incidence and prevention. Lancet **1**: 807-812.

Koch, M. L., D. Lepley, Jr., C. M. Schroeder, and M. B. Smith. 1959. Study of staphylococcic infections occuring on a surgical service. J. Am. Med. Assoc. **169**:83-88.

Klarmann, E. G. 1959. Hospital infection and environmental disinfection. Maintenance & Sanitary Supplies **4**:

Klarmann, E. G. 1960. Disinfectants and antiseptics. Med. Chem. **2**:1109-1142.

Lawrence, C. A. 1958. The effects of disinfectants on antibiotic resistant and antibiotic sensitive strains of Micrococcus pyogenes, var. aureus. Surg. Gynecol. Obstet. **107**:679-684.

McLean, J. C. 1961. The control of nosocomial infections. Hospital Management.

Mudd, S. 1958. Conference on staphylococcic infections. J. Am. Med. Assoc. **166**:1177-1204.

Nahmias, A. J., and T. C. Eickhoff. 1961. Staphylococcal infections in hospitals. New Engl. J. Med. **265**:177-182.

Vogel, R. A., Y. J. Trotter, J. H. Shinaberger, and J. E. McCroan. 1959. The genesis and spread of a hospital staphylococcal epidemic on an adult medical ward. New Engl. J. Med. **261**: 1302-1310.

Walter, C. W., and R. B. Kundsin. 1960. The floor as a reservoir of hospital infections. Surg. Gynecol. Obstet. **111**:412-422.

Wise, R. I., F. J. Sweeney, Jr., G. J. Haupt, and M. A. Waddell. 1959. The environmental distribution of Staphylococcus aureus in an operating suite. Ann. Surg. **149**:30-42.

Wysham, D. N., and W. M. M. Kirby. 1957. Micrococcic (staphylococcic) infections in a general hospital. J. Am. Med. Assoc. **164**:1733-1739.

# PHARMACOLOGY, METABOLISM, AND ANTISEPTIC EFFECTS OF TWO PROPOCTAMINE COMPOUNDS

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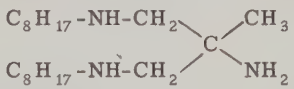
Preliminary pharmacological and clinical data on two propoctamine compounds have been presented elsewhere (Mulinos, 1961; Mulinos and Erger, 1961). Chemical specifications of this group of compounds have been published by Senkus (1961). This report expands upon the chemical and physical properties of these drugs, and includes complete pharmacological and clinical studies and $C^{14}$ tracer data on the metabolism of one of the compounds.

## Materials and Methods

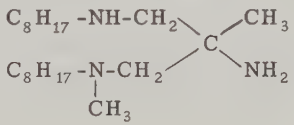
*Chemical and physical properties.* The generic name of propoctamine has been chosen for these compounds because of their chemical content of two octyl chains (ethylhexyl) joined to propyltriamine. A similar group of compounds in the ring structure of hexahydropyrimidine, with analogous antimicrobial properties (New and Non-Official Drugs, 1961) had been reported previously (Senkus, 1945*a*, *b*, 1946). Propoctamine is:

2-methyl-$N^1$,$N^3$-bis(2-ethylhexyl)-1,2,3-propanetriamine



and methylpropoctamine is:

$N^1$-2,dimethyl-$N^1$,$N^3$-bis(2-ethylhexyl)-1,2,3-propanetriamine



The propoctamines are liquid alkaloids which are insoluble in water, but form water-soluble salts with mineral and organic acids, and soaps with fatty acids. They are about equally potent antimicrobial agents, effective against a wide spectrum of bacteria, fungi, yeasts, protozoa, and various insect grubs. Their strongly substantive adherence to the skin and various fabrics suggested their use in accessible topical infections due to susceptible organisms, and in industry for the protection of fabrics against various destructive organisms, including the clothes moth.

## Results

*Toxicological studies.* Tables 1 and 2 show the low order of toxicity of the propoctamines. $C^{14}$ tracer studies indicate a rapid metabolic degradation of these chemicals, which may account for the lack of cumulative action from daily dosing. From oral dosing, sedation was the rule, but this was never more than quieting to the

TABLE 1. *Acute toxicity of two antimicrobial propoctamines in the rat*[a]

| | Propoctamine | | Methyl-propoctamine | |
|---|---|---|---|---|
| Toxicity | Oral | Intra-peritoneal | Oral | Intra-peritoneal |
| *mg/kg* | | | | |
| $LD_{100}$ | 1,350 | 116 | 1,500 | 130 |
| $LD_{50}$ | 800 | 100 | 1,000 | 115 |
| $LD_0$ | 500 | 55 | 550 | 70 |

[a]The drugs were dissolved in corn oil and administered in equal volume to adult rats of 125 to 150 g.

TABLE 2. *Subacute toxicity of propoctamine antiseptics*[a]

| Compound | Weekly body wt (g) | | | | | Hb[c] |
|---|---|---|---|---|---|---|
| | Initial | 1 | 2 | 3 | 4[b] | |
| | | | | | | % |
| Propoctamine | 102 (5)[d] | 114 (5) | 116 (4) | 117 (4) | 114 (3) | 70, 80, 75 |
| Methyl-propoctamine | 102 (5) | 113 (5) | 116 (4) | 112 (3) | 110 (2) | 80, 75 |
| Controls | 101 (5) | 120 (5) | 135 (5) | 146 (5) | 160 (5) | 80, 85, 85 |

[a]Administered daily in 250 mg/kg oral doses dissolved in oil.

[b]Surviving animals were sacrificed after 32 to 37 daily drug administrations.

[c]Sahli test on cardiac blood.

[d]Figures in parentheses represent the number of rats used.

animals and lasted only from 1 to 2 hr. Lethal oral doses resulted in progressive depression and immobility without restlessness. The animals died in a sitting position. A loss in weight was accompanied by a diminution in food intake, but death was not due to starvation. After 21 to 37 days of daily oral administration, the surviving animals were autopsied and their coded tissues examined by a pathologist. No specific lesions attributable to the medication were found, and it was not possible to determine the reason for death or for the gradual loss in weight when compared to the controls.

Adult rabbits received a slow intravenous dose of 10 mg/kg methylpropoctamine hydrochloride, as a 0.1% solution in saline. There were tetanic convulsions 5 to 10 min after the injection, from which the animals recovered promptly. Repetition of the dose on the following day elicited the same reaction, with prompt recovery. Death, when it occurred, was due to injury from the violence of the convulsions, and not attributable to the drug.

Mice were used for the determination of the acute toxicity of propoctamine hydrochloride and methylpropoctamine hydrochloride, dissolved in saline (Table 3). Due to the local binding by the tissues and the more gradual release, the drug was much less toxic by the intramuscular and subcutaneous routes than when given intraperitoneally.

TABLE 3. *Acute toxicity of two propoctamines in the mouse*

| | Propoctamine HCl | | | | Methylpropoctamine HCl | | | |
|---|---|---|---|---|---|---|---|---|
| Toxicity | Ip | Im | Sc | Oral[a] | Ip | Im | Sc | Oral[a] |
| *mg/kg* | | | | | | | | |
| $LD_{100}$ | 51.0 | >3,000 | >3,000 | 1,600 | 34.8 | 3,000 | 3,000 | 1,400 |
| $LD_{50}$ | 38.3 | 2,000 | 2,500 | 1,160 | 25.8 | 2,000 | 2,000 | 1,055 |
| $LD_0$ | 25.6 | 1,000 | 1,500 | 800 | 17.2 | 1,000 | 1,000 | 800 |

[a]Alkaloid dissolved in oil.

Toxicity from percutaneous absorption of the propoctamines was determined on rabbits by the daily application of a vanishing cream type of ointment containing up to 20% of each drug. From the daily application of the 20% strength, the skin was hardened, forming a sterile, scab-like surface which was easily removed, revealing a normal skin underneath. There was loss in weight after 90 daily applications of the ointment in a dose of 1.0 g/kg body weight, equivalent to 200 mg/kg of drug. Autopsy revealed no tissue damage to account for the loss in weight. The blood count and hemoglobin concentration remained unchanged throughout this period.

*$C^{14}$ tracer studies.* $C^{14}$ tracer studies were performed with methylpropoctamine, in which the $N^1$-methyl carbon was the radioactive isotope, $C^{14}$. A dose of 20 mg/kg of methylpropoctamine as the hydrochloride, containing a total of 10.79 $\mu$c of radioactivity, was

injected subcutaneously in saline into an adult male rat, which was immediately placed in the metabolism chamber. Samples of respiratory $CO_2$ were collected hourly for 27 hr, when the animal was sacrificed.

The respiratory $CO_2$ contained a total of 3.798 $\mu$c of radioactivity, representing 41.5% of total recovery. Actually, this was 74% of the amount absorbed from the site of injection. There was 0.0955 $\mu$c of $C^{14}O_2$ activity collected during the first hour, reaching a peak of 0.2725 during the third hour, then falling very slowly to 0.1376 during the 12th hour, and to 0.0983 during the 27th hour. Table 4 shows the distribution of $C^{14}$ activity in the tissues, urine, and feces at autopsy, which accounted for 85% of the injected activity.

TABLE 4. *Distribution of $C^{14}$ activity of methylpropoctamine hydrochloride in the tissues of an adult 250-g male rat*[a]

| Source | Activity of $C^{14}$ recovered | | Specific activity |
|---|---|---|---|
| | $\mu$c | % | $\mu$c/g |
| Total injected | 10.790 | 100 | $2.813 \times 10^{-2}$ |
| Total recovered | 9.173 | 85 | $2.411 \times 10^{-2}$ |
| Expired air in 27 hr | 3.798 | 41.5[b] | – |
| Tissue at site of injection | 4.047 | 44.2 | $7.075 \times 10^{-2}$ |
| Carcass | 0.240 | 2.6 | $8.325 \times 10^{-4}$ |
| Liver | 0.054 | – | $3.673 \times 10^{-3}$ |
| Kidneys | 0.017 | – | $5.000 \times 10^{-3}$ |
| Lungs | 0.009 | – | $6.100 \times 10^{-3}$ |
| Heart | 0.008 | – | $4.710 \times 10^{-3}$ |
| Gastrointestinal tract | 0.485 | 5.5 | $2.526 \times 10^{-2}$ |
| Feces | 0.034 | – | – |
| Urine | 0.481 | 5.2 | – |

[a]Dosage was 20 mg/kg administered subcutaneously.
[b]Expressed as percentage of the 9.173 $\mu$c recovered.

The tissue into which the injection had been made contained 44.2% of the recovered $C^{14}$ activity, with a specific activity of 7.075 $\mu$c/g, which was almost 100 times greater than in the remaining carcass. The high concentration of methylpropoctamine still remaining at the site of injection after 27 hr can be explained by a binding of the drug by the tissues. This surmise is supported by the much lower toxicity of methylpropoctamine after subcutaneous and intramuscular injection, compared to an intraperitoneal injection (Table 3).

The percutaneous absorption of $C^{14}$ methylpropoctamine was tested in the form of an ointment containing 5.0% of the tricaprylate soap.

The ointment (1 g), containing 5.83 $\mu$c of $C^{14}$ activity, was spread evenly over a 4 × 4 cm area of shaved skin on the back of an adult rat, adequately restrained to prevent loss of ointment by licking or contact with the cage. During the following 22 hr, no $C^{14}O_2$ appeared in the expired air, indicating no absorption.

The skin was washed with acetone containing dilute hydrochloric acid. The washings contained 5.14 $\mu$c or 88% of the total applied to the skin. The skin itself contained 0.06 $\mu$c of $C^{14}$ activity.

Local irritant properties of the propoctamines were tested on skin of rabbits and rats, and on the conjunctiva and prepuce of rabbits. The alkaloids dissolved in oil in concentrations of 50% or more caused moderate to severe conjunctival injury, with occasional corneal opacities. However, concentrations of 20% resulted in redness and some edema, but no corneal or conjunctival injury.

The prepuce membranes of the rabbit were not injured by concentrations of up to 20%, indicating a special reactivity of the conjunctival membranes to the propoctamines.

The human skin was not affected when the pure alkaloids were rubbed into it in small amounts. When a small droplet of the pure propoctamine was placed on the tongue or the inner surface of the lips, there was a prickling sensation, reminiscent of tincture of aconite, but no evidence of an inflammatory reaction developed.

Patch tests for primary irritation were performed on 100 subjects, using a 1.0% concentration in Hydrophilic Ointment, USP. After 48 hr the skin showed no "immediate" reaction, nor any "immediate delayed" reaction 15 min later, and 14 days later a provocative patch test failed to reveal the development of allergic sensitization.

Another 50 subjects were subjected to test patches impregnated with each of four ointments containing 5% of the propionate or caprylate salt of propoctamine and methylpropoctamine. The procedure was followed every 48 hr for nine applications, as recommended by Shelanski (1951), without eliciting any evidence of primary irritation. Following a 2-week rest period, a final provocative patch test with each of the ointments was also negative.

Further evidence of the nonirritant and nonallergenic properties of a 1.0% ointment of the propoctamines was obtained on 25 female subjects. The ointments were applied daily for 4 weeks as underarm deodorant preparations, without a single instance of irritation. Effective deodorization, but no antiperspirant effect, was reported by most of the subjects, who compared the ointments with their personal choice for this purpose. Another 25 female subjects applied the propoctamine ointments to the scalp daily for 1 month, without evidence of irritation. After a rest period of 2 weeks, both the axilla use and scalp use subjects received a provocative patch test, without any evidence that sensitization had occurred. It was concluded that the propoctamines were nonirritant and nonallergenic, and could be used topically for the treatment of skin infections.

*Antimicrobial properties.* The in vitro antimicrobial activities of the propoctamines are shown in Table 5. The broad-spectrum antimicrobial potencies of the compounds extend to fungi and other organisms destructive to fabrics and wool.

TABLE 5. *Antimicrobial spectrum of the propoctamines*

| Organism | Minimal inhibitory concentration | |
|---|---|---|
| | Propoctamine | Methylpropoctamine |
| | *μg/ml* | *μg/ml* |
| *S. aureus* | 1-5 | 1-5 |
| *S. faecalis* | 1-5 | 1 |
| *S. pyogenes* | 1-5 | 1-5 |
| *P. pseudotuberculosis* | 5-10 | 15-25 |
| *B. subtilis* | <1 | <1 |
| *C. xerose* | 1-5 | <1 |
| *K. pneumoniae* | 50-100 | 50-100 |
| *E. coli* | >200 | >200 |
| *S. dysenteriae* | >200 | 100-200 |
| *S. typhosa* | 100-200 | 50-100 |
| *P. aeruginosa* | >200 | >200 |
| *A. aerogenes* | 200 | 200 |
| *P. vulgaris* | >200 | >200 |
| *S. typhimurium* | >200 | >200 |
| *A. niger* | 500-1,000 | 100-500 |
| *C. albicans* | 100-500 | 100-500 |
| *P. chrysogenum* | 100-500 | 100-500 |
| *F. oxysporum* | 100-500 | 100-500 |

Tests were made to determine the degree of protection of cotton duck fabric, treated with the chemicals, against the destructive effects of soil bacteria and mildew-producing organisms. The treated fabric strips were buried in moist soil kept at room temperature. Control, untreated cotton fabric strips showed complete loss of strength after a standard 2-week period (Table 6). Similar protection

TABLE 6. *Soil burial treatment tests and loss in strength of cotton duck fabric strips*[a]

| Compound | Concentration in acetone | Loss in strength |
|---|---|---|
| | % | % |
| Propoctamine | 0.05 | 75 - 90 |
| | 0.10 | 40 - 50 |
| | 0.50 | 0 |
| Methylpropoctamine | 0.05 | 75 - 90 |
| | 0.10 | 40 - 50 |
| | 0.50 | 0 |
| Control | 0 | 90 - 100 |

[a]The fabric strips were immersed in the acetone solution of each compound and dried prior to burial in the soil.

was afforded against slime and mold organisms on cotton fabric immersed in a circulating water system until there was complete loss of strength in the control, untreated fabric strips.

The propoctamines possess many of the chemical and physical qualities which have been found essential for effective fungicidal and bactericidal activity. According to McNew (1960), an effective antimicrobial agent must have four attributes, in addition to those of systemic safety and freedom from irritation and allergenicity. (i) It must penetrate the cell membrane, in order to become established as a site of operation. (ii) It must react with cell metabolites and disrupt the biochemical processes of the cell essential to its growth and development. (iii) It must be selective and not enter into extraneous reactions, become detoxified, or get attached to inert structures such as the spore wall. (iv) It must be stable for proper dispensing in suitable vehicles from which it can penetrate into the cell and be decomposed into antiseptic congeners. If it is not sufficiently lipoid-soluble, it will not reach the cell membrane, and if it lacks hydrophilic properties, it will not transfer from the cell membrane to the cytoplasm (Miller and McCallan, 1957).

Wellman and McCallan (1946) found that the fungal toxicity of a series of imidazoline derivatives increased with elongation of the side chain up to the 17-carbon compound, then diminished as the chain was lengthened beyond 17-carbons. McNew (1960) felt that the increasing fungitoxic effect with lengthening of the side chain was due to progressively increased lipoid solubility which allowed the compounds to penetrate the spores of the fungus.

## Discussion

The propoctamines have two equal side chains of 8-carbons (ethylhexyl), and their fungitoxic activity may be due to their lipoid-solubility which is complete, although their affinity for anionic radicals within the cell can render them sufficiently hydrophilic to fulfill the criteria for antimicrobial activity. Increasing the hydrophilic properties of these compounds by combination with aliphatic acids also increases their fungitoxic activity.

The antimicrobial spectrum of the propoctamine derivatives indicates a high degree of activity in vitro, against a wide variety of organisms (Table 5).

*Clinical studies.* It was recognized at the outset that the clinical effectiveness of any topically applied remedy against local infections depends upon the susceptibility of the infecting organism as well as the pathological process with which it is involved, because the latter may protect the organisms from ready contact with the remedy. This is the case with deep-seated infections due to *Candida* or *Microsporum audouini*, and the small bacterial abscesses such as those

which occur in acne. The ultimate criterion of utility can be determined only by trial under actual clinical conditions.

Among 41 cases with fungus infections on various areas of the scalp and body, 28 or 68% were cured (Table 7). However, the failures were all among three organisms which are notoriously resistant to treatment, irrespective of in vitro sensitivity of the organisms. These were *M. audouini*, *Trichophyton rubrum*, and *Candida albicans*. The higher concentrations of ointment were no more effective than 1.0%, indicating maximal activity at this concentration (Table 7).

TABLE 7. *Clinical use of methylpropoctamine*[a]

| Cases | Number | Result |
|---|---|---|
| Fungal | | |
| *E. flocculosum* | 9 | Cured |
| *M. furfur* | 2 | Cured |
| *M. lanosum* | 6 | Cured |
| *T. gypseum* | 10 | Cured |
| *T. mentagrophytes* | 1 | Cured |
| *M. audouini* | 2 | No effect |
| *T. rubrum* | 5 | No effect |
| *C. albicans* | 6 | No effect |
| Bacterial | | |
| Staphylococcal impetigo | 8 | Cured |
| Staphylococcal or streptococcal infections | 7 | Cured |
| Mixed, sycosis barbae | 1 | Cured |
| Mixed, sycosis barbae | 4 | No effect |
| Mixed, plus *E. coli* | 3 | No effect |

[a]Used as 1.0 to 5.0% ointments.

The infecting fungi were recovered mostly from the scalp and feet of these patients. Of the six *C. albicans* infections, four were associated with chronic paronychia and were resistant to other therapy as well. In all cases the infecting organism was confirmed by culture, and the criteria for cure were both clinical relief and persistent absence of the infecting organisms from scrapings of the skin.

Of 23 culturally confirmed bacterial infections, 15 which were associated with staphylococci and streptococci yielded readily to treatment with the ointment. Where the bacteria were not accessible to the ointment, as in one furuncle and two cases of acne, it proved to be of little value. Four of five cases of sycosis barbae with mixed infection failed to improve. Impetiginous lesions responded promptly and well. At no time was there any evidence of irritation to the skin which could be ascribed to the therapy.

## Literature Cited

McNew, G. L. 1960. Fungicides and bactericides for controlling plant diseases. A. A. A. S. Publication no. 61.

Miller, L. P., and S. E. A. McCallan. 1957. Movement of ions and compounds into and out of fungus spores. Science **126**:1233.

Mulinos, M. G. 1961. Pharmacology of the triamines. First International Pharmacological Meeting, Stockholm.

Mulinos, M. G., and B. Erger. 1961. Pharmacology and antiseptic effects of two propoctamine compounds. Pharmacologist **3**:78.

New and nonofficial drugs. 1961. J. B. Lippincott Co., Philadelphia, p. 157.

Senkus, M. 1946. The preparation of some hexahydropyrimidines from nitroparaffins. J. Am. Chem. Soc. **68**:1611-1613.

Senkus, M. 1945*a*. Preparation of some hexahydropyrimidines. U. S. Patent no. 2,387,043.

Senkus, M. 1945*b*. Preparation of some hexahydropyrimidines. U. S. Patent no. 2,391,847.

Senkus, M. 1948. Triamino propanes. U. S. Patent no. 2,436,779.

Shelanski, H. A. 1951. Experiences with and consideration of the human patch test method. J. Soc. Cosmetic Chemists **2**:324-331.

Wellman, R. H., and S. E. A. McCallan. 1946. Glyoxaline derivatives as foliage fungicides. I. Laboratory studies. Contribs. Boyce Thompson Inst. **14**:151-160.

# INVESTIGATION OF TISSUE DISTRIBUTION OF SULFAMOXOLE AND 2-(*p*-AMINOBENZENE SULFONAMIDO)-5-METHOXYPYRIMIDINE

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The evaluation of the therapeutic value of a new sulfonamide is dependent on a number of parameters. For a particular compound, the specific antibacterial activity and various physicochemical properties such as degradation, chemical modification, acetylation, protein binding, and distribution between serum and tissue have to be considered.

The problem of distribution is of primary importance because the maximal blood or serum levels of a drug such as sulfonamide are a poor index of its chemotherapeutic efficacy, since the absolute values of the blood or serum concentrations differ from sulfonamide to sulfonamide. Therefore, a knowledge of the distribution of a sulfonamide in the body under a variety of pathological conditions is fundamental.

Our experimental investigations are concerned with the problem of serum concentration that can be achieved when sulfamoxole or sulfadimethoxidiazine are administered in a regular clinical dosage schedule, either as single or repeated doses. We determined what concentrations can be achieved in normal and pathologically altered tissue when equal doses are administered in either case.

## Materials and Methods

The method of Schmidt and Padowetz (1957), as modified by Traut and Kuemmerle (1960), was used for the determinations of the sulfonamide levels. The tissues were prepared according to Egger (1946), and the free and total sulfonamide was determined in the filtrate by the method of Traut and Kuemmerle.

Protein binding in serum was determined, before and after dialysis, with the method of Scholtan (1961). Five sets of experiments were performed on 92 patients.

The tissue concentrations were determined as follows. Tissue was excised and blood withdrawn at the same time during the operation

(12 to 14 hr after a single 2-g oral dose of sulfamoxole or 1- and 2-g doses of sulfamethoxidiazine, respectively); the coefficient for the distribution between tissue and serum was then calculated for the individual tissues.

## Results

Our investigations show that sulfamoxole is a typical sulfonamide with an intermediate half-life, whereas sulfamethoxidiazine is a typical long-acting drug.

Sulfamoxole reaches its maximal serum concentrations after 4 hr with 12.9 free and 14.9 mg per 100 ml total sulfonamide. After 24 hr the values have decreased to 3.6 and 4.6 mg/100 ml, respectively (Fig. 1).

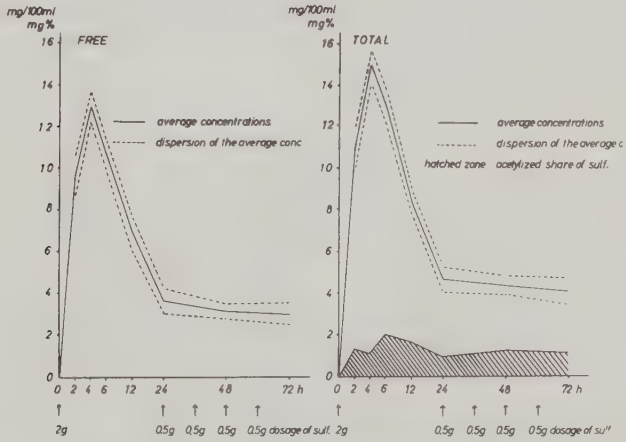


FIG. 1. *Average serum concentrations of free and acetylized sulfamoxole after repeated oral administration in 22 human subjects.*

The biological half-life ($t_{50\%}$) is 7 hr, 53 min. The average percentage of protein-bound free sulfonamide is 27.36%. The degree of acetylation averages 20.85% of the total sulfonamide concentration.

Sulfamethoxidiazine has the following maximal serum concentration at 6 hr after a 1-g dose: 9.6 free and 10.2 mg/100 ml total (Fig. 2).

After 24 hr the concentrations are 6.10 and 7.2 mg/100 ml, respectively. The half-life is 29 hr, 34 min.

After a 2-g dose maximal levels were reached after 4 hr with 16.8 and 17.1 mg/100 ml, respectively (Fig. 3). The half-life in this case was 43 hr, 2 min.

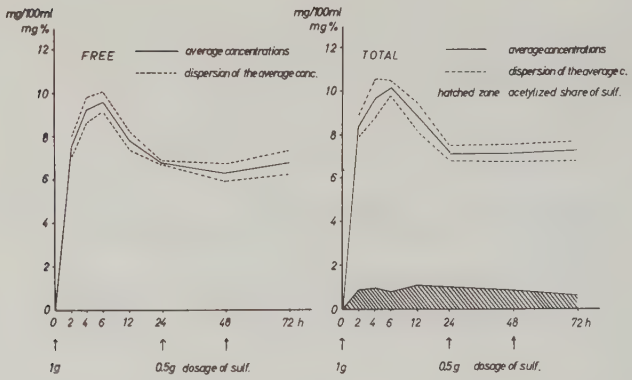


FIG. 2. *Average serum concentrations of free and acetylized sulfamethoxidiazine after repeated oral administration in 20 human subjects with an initial dose of 1 g.*

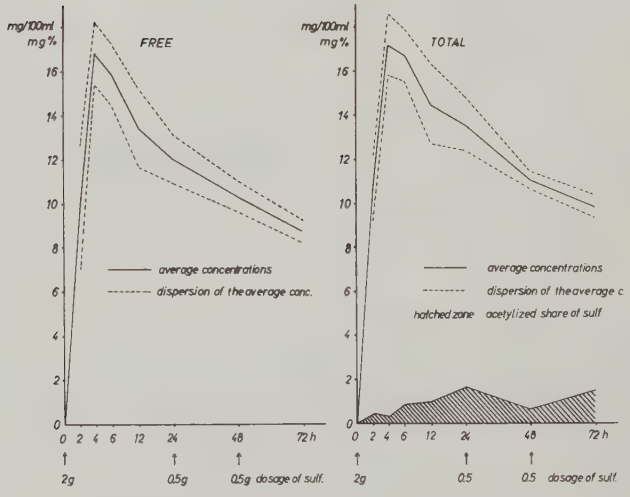


FIG. 3. *Average serum concentrations of free and acetylized sulfamethoxidiazine after repeated oral administration in 3 human subjects with an initial dose of 2 g.*

The quantity of protein which is dialyzed averages 22.35%, and the drug was acetylated to the extent of 9.67% after the 1-g dose and 6.9% after the 2-g dose.

*Tissue concentrations in pathologically altered tissue.* The tissue specimens were, on the average, 0.40 free and 0.42 total sulfonamide with average serum concentrations of 12.4 and 14 mg/100 ml, respectively.

For sulfamethoxidiazine the corresponding values, obtained from 63 tissue specimens, were as follows: 0.41 and 0.42 at serum concentrations of 18.2 and 21.9 mg/100 ml, respectively.

Remarkably high values of distribution coefficients were found for malignant tumors as well as for vagina, parametrium, umbilical cord, appendix, tube, cervix, portio, and muscles (Fig. 4).

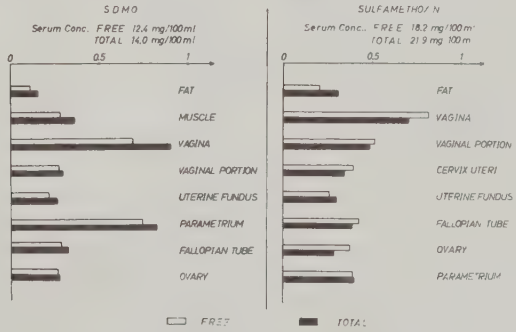


FIG. 4. *Mode of drug distribution in various types of tissue.*

Relatively high values were found for the ascites, cystic fluid of ovarian cysts, connective tissue, vascular tissue, and tumor casing. The low concentrations in fatty tissue are noteworthy.

## Discussion

The investigation clearly demonstrates that sulfamoxole is a typical sulfonamide with intermediate half-life and sulfamethoxidiazine is a typical long-acting sulfa drug.

A comparison of efficacy of the two sulfonamides is, therefore, impossible. Consequently, because of the difference in their properties, the two drugs are applicable in different areas. The two compounds do not differ significantly with respect to their protein binding. As emphasized in previous publications (Kuemmerle, 1960; Kuemmerle and Traut, 1960; Kuemmerle and Contzen, 1961), the magnitude of the blood or serum concentrations does not provide a reliable indicator of the therapeutic efficacy of a sulfonamide. On the basis of

extensive experimental work, we have come to essentially the same conclusion for antibiotics and shall report these findings elsewhere.

Modern chemotherapy has recognized that tissue concentrations or distribution coefficients between tissue and serum, respectively, are the only important and reliable basis for the evaluation of a new sulfonamide. Tissue distribution coefficients provide an exact and reliable criterion for judging the efficacy of a sulfonamide.

The distribution coefficient is generally below 1, averaging 0.40 to 0.45. The drugs do not generally accumulate in tissues. The highest tissue concentrations for the two sulfonamides have been found in malignant tumors and vaginal tissue. In this respect they resemble the antibiotics. This observation is explained by the higher degree of penetration exhibited by these drugs, as well as the differences in permeability shown by the cells of the normal or pathologically altered tissue.

Diffusion, therefore, is the critical factor for the magnitude of tissue concentration and, consequently, for the therapeutic efficacy. Vascular diffusion is a necessary process; it is, however, only of secondary importance.

## Literature Cited

Egger, P. 1946. Die Verteilung der Sulfonamide im Organismus. Benno Schwabe und Co., Basel.

Kuemmerle, H. P. 1960. Klinische Pharmakologie und Toxikologie der Sulfonamide in Kuemmerle, Senn, Rentchnick, Goossens" Klinik und Therapie der Nebenwirkungen ". G. Thieme, Stuttgart.

Kuemmerle, H. P., and H. Contzen. 1961. Comparative clinical and experimental investigations concerning tissue and serum-concentrations of demethylchlortetracycline and pyyrolidinomethyltetracycline under pathological conditions in man. Antimicrobial Agents Ann. 1961, p. 202-205.

Kuemmerle, H. P., and H. Traut. 1960. Klinische-experimentelle Untersuchungen zum Problem des Sulfonamid-Kombinations prinzips mit der Sulfonamid-Kombination SH 522-2 und vergleichende Betrachtungen zu anderen Sulfonamiden. Chemotherapia 1:167-174.

Schmidt, K., and J..Padowetz. 1957. Hausmitteilungen der Firma CIBA AG., Basel, June 25.

Scholtan, W. 1961. Die Bindung der Langzeitsulfonamide an die Eiweisskörper des Serums. Drug Research 11:681-739.

Traut, H., and H..P..Kuemmerle. 1960. Kritische Betrachtungen zur Methodik der Bestimmung von freiem und gebundenem bzw. Gesamit-Sulfonamid im Blut. Chemotherapia 1:175-182.

# ADSORPTION OF BIOLOGICAL AGENTS TO COMPONENTS IN SERUM AND IN WHOLE BLOOD

## I. Sulfonamides

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Numerous studies of adsorption to serum proteins have been published in the past. Since Cushny (1920) used collodion bags, first introduced by Walpole (1915), most adsorption studies have been performed with semipermeable membranes. Loken et al. (1960) described an ultracentrifugation method for analyses of protein-bound and free calcium in serum. This paper reports the extent of binding of some sulfonamides to protein and cellular constituents in human whole blood as determined by the ultracentrifugation method.

The binding of sulfonamides to proteins has been reported by many investigators. A relation between long-acting sulfonamides, protein-binding, and efficacy has been discussed by Anton (1960) and Newbould and Kilpatrick (1960). This report adds the important factor of blood cells in adsorption studies with sulfonamides.

### Materials and Methods

Each sulfonamide was dissolved in 0.1 M potassium phosphate buffer (pH 7.9) to a concentration of 1 mg/ml. Duplicate samples of 0.9 ml were added to Spinco 9-ml tubes. Either pooled human serum or fresh human whole blood (8.1) was added to each tube, resulting in sulfonamide concentrations of 100 $\mu$g/ml of serum or whole blood. The solutions were stirred and the tubes placed in a Spinco 30.2 head and centrifuged at 30,000 rev/min and 0 C for 24 hr. With this angular-type head (14°), the centrifugal force was 63,000 × $g$ at the top of the tube, 79,000 × $g$ at the center, and 94,500 × $g$ at the bottom.

After centrifugation, the upper 1.5-ml portion of each serum and whole blood sample was removed for assay. The remaining 7.5 ml of each serum sample and the remaining liquid in each whole blood

sample were mixed and also assayed. The blood clot was removed and ground to a liquid state in a tissue grinder. All samples were assayed for sulfonamide content by the method of Bratton et al. (1939).

## Results and Discussion

The concentrations of sulfonamide in the upper layers of serum and whole blood after centrifugation are shown in Table 1. The extent of binding was calculated for the serum only. If the assumption is made that protein-bound substances remain bound under the force of ultracentrifugation, then the sulfonamide in the upper, water-clear layer is in the free form. Therefore, the percentage of protein-bound sulfonamide at an initial concentration of 100 μg/ml was 100 minus the concentration in the upper layer, with a correction factor for serum protein volume considering that the protein was removed. Table 1 shows variations in percentage binding among the sulfonamides from 63 to 93%.

TABLE 1. *Analyses of upper layers of blood after ultracentrifugation and the relation to serum protein binding*

| Sulfonamide | Concentration in upper layers, μg/mg* | | | | | |
|---|---|---|---|---|---|---|
| | Whole blood, actual | | Serum | | | |
| | | | Actual | | Correction, percentage free† | Binding to serum proteins |
| | | | | | | % |
| 2-sulfanilamidopyrimidine (Sulfadiazine) | 57 | 51 | 36 | 39 | 35 | 65 |
| 2-sulfanilamidothiazole (Sulfathiazole) | 25 | 26 | 18 | 18 | 17 | 83 |
| 3,4-dimethyl-5-sulfonamido-isoxazol (Sulfisoxazole) | 15 | 15 | 12 | 21 | 16 | 84 |
| 2,4-dimethoxy-6-sulfanilamido-1,3-diazine (Sulfadimethoxine) | 7 | 8 | 7 | 9 | 7 | 93 |
| 3-sulfanilamido-6-methoxypridazine (Sulfamethoxypyridazine) | 6 | 3 | 9 | 9 | 8 | 92 |
| 2-*p*-aminobenzolsulfonamido-4,5-dimethyloxazol (Sulfamoxole) | 21 | 20 | 11 | 17 | 13 | 87 |

*Original concentrations were 100 μg sulfonamide per ml of whole blood or serum. Results of two experiments are recorded.

†Correction factor (0.93) for serum protein volume (Loken et al., 1960).

The percentage of binding to whole blood was not estimated, because of insufficient data concerning the effect of the cells and fibrin in the system. This is presently being investigated and will be reported at a later date. However, data with whole blood are included to show that the system with cells is possible.

The results of the experiments with sulfamoxole are shown in Table 2. The original concentration of sulfonamide was 100 μg per ml of serum or whole blood. Since the serum in the stationary control with the whole blood experiments assayed 96 μg/ml, it is assumed that the sulfonamide was distributed approximately evenly between the serum and the cells. However, not all of the drug was recovered from the blood clot. The recovery was nearly complete with all the sulfonamides.

TABLE 2. *Concentration of sulfamoxole in serum and whole blood after ultracentrifugation*

| Location of sulfamoxole | Tube | Layer | Vol. | Concentration | Total | Recovery |
|---|---|---|---|---|---|---|
| | | | *ml* | *μg/ml* | *μg* | % |
| Serum | 1 | Upper | 1.5 | 10 | 15 | |
| | | Lower | 7.5 | 108 | 810 | |
| | | Total | 9.0 | | 825 | 92 |
| | 2 | Upper | 1.5 | 16 | 24 | |
| | | Lower | 7.5 | 108 | 810 | |
| | | Total | 9.0 | | 891 | 93 |
| | Control (not centrifuged) | Serum | 9.0 | 102 | 918 | 102 |
| Whole blood | 1 | Upper | 1.5 | 21 | 32 | |
| | | Pooled liquid remaining | 3.5 | 198 | 693 | |
| | | Blood clot | 4.0 | 42 | 168 | |
| | | Total | 9.0 | | 893 | 99 |
| | 2 | Upper | 1.5 | 20 | 30 | |
| | | Pooled liquid remaining | 3.5 | 198 | 693 | |
| | | Blood clot | 4.0 | 48 | 192 | |
| | | Total | 9.0 | | 915 | 102 |
| | Control (not centrifuged) | Serum | 4.5 | 96 | 432 | |
| | | Blood clot | 4.5 | 78 | 351 | |
| | | Total | 9.0 | | 783 | 87 |

A biuret assay on the upper layer of ultracentrifuged serum indicated that a concentration of approximately 1% of the protein remained in the upper 2-ml layer, relative to the uncentrifuged serum, and the lower 2-ml layer assayed approximately 250%. Throughout these studies, serum with lipid-rich opalescent top layers was not used, but light lipid layers, when noted, were avoided in the sampling.

A comparison of results of serum binding found by ultracentrifugation and those reported in the literature is shown in Table 3. It can be seen that the comparative results are relatively close, considering the differences in methods among investigators.

The ultracentrifugation method of testing for binding to protein or cells has some distinct advantages over the usual dialysis procedure. It is more efficient, and the cell system can readily be

included without introducing salts, as is required in dialysis with isotonic solutions.

TABLE 3. *Comparison of binding determined by ultracentrifugation methods with literature reports*

| Sulfonamide | Bound in serum* | | Literature reference |
|---|---|---|---|
| | Ultracentrifugation | Literature | |
| | % | % | |
| Sulfadiazine | 65 | 55 | Davis (1942) |
| Sulfathiazole | 83 | 75 | Davis (1942) |
| Sulfisoxazole | 84 | 84 | Anton (1960) |
| Sulfadimethoxine | 93 | – | – |
| Sulfmethoxypyridazine | 92 | 83 | Anton (1960) |
| Sulfamoxole | 88 | 73 | Kuemmerle, Dimpfl, and Roettger (1962) |

*All tests except for sulfamoxole were made with 100 μg sulfonamide per ml serum.

## Acknowledgment

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## Literature Cited

Anton, A. 1960. Relation between the binding of sulfonamides to albumin and their antibacterial efficacy. J. Pharmacol. Exptl. Therap. **129**:282-290.

Bratton, A. C., E. K. Marshall, Jr., D. Babbitt, and A. R. Henduckson. 1939. A new coupling component for sulfanilamide determination. J. Biol. Chem. **128**:537-550.

Cushny, A. R. 1920. Collodian membranes for ultrafiltration and pressure dialysis. J. Physiol. (London) **53**:391-398.

Davis, B. D. 1942. Binding of sulfonamides by plasma proteins. Science **95**:78.

Kuemmerle, H. P., P. Röttger, and J. Dimpfl. 1962. Investigation of tissue distribution of sulfamaxole and 2-(*p*-aminobenzene sulfanamido)-5-methoxypyrimidine. Antimicrobial Agents and Chemotherapy - 1961, p. 866-870.

Loken, H. F., R. J. Havel, G. S. Gordon, and S. L. Whittington. 1960. Ultracentrifugal analysis of protein-bound and free calcium in human serum. J. Biol. Chem. **235**:3654-3658.

Newbould, B. B., and R. Kilpatrick. 1960. Long-acting sulfonamides and protein binding. Lancet **1**:887-891.

Walpole, G. S. 1915. The colloid-free filtrate of serum. Biochem. J. **9**:284-297.

# EFFECTS OF ETHYL MERCAPTAN COMPOUNDS IN MURINE LEPROSY

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Anderson and Chin (1947) found that 2,3-dimercaptopropanol inhibited growth of *Mycobacterium tuberculosis* in vitro. Del Pianto (1950, 1953) reported the chemotherapeutic activity of sodium ethylthiosulfate in experimental tuberculosis of guinea pigs and mice and in a considerable number of cases of human tuberculosis. Recently, this drug has been claimed to be effective in the treatment of leprosy (del Pianto, 1958).

Brown et al. (1954) reported that of more than 350 samples of mercaptan compounds examined over 50 showed antituberculosis activity in mice. Among these samples S-ethyl-L-cysteine was worthy of consideration on the basis of efficacy and of low toxicity. When submitted to other investigators for trial in guinea pigs and mice, this compound was found inactive when the animals were kept in open cages, but of high activity when closed jars were used. Evidently S-ethyl-L-cysteine was converted into a volatile component which was active against tubercle bacilli in vivo; this component was demonstrated to be ethyl mercaptan (Solotorovsky, Ironson, and Winton, 1956).

Mathewson, Thoren, and Hinshaw (1956) treated 23 patients suffering from various types of tuberculosis with S-ethyl-L-cysteine. Symptomatic, roentgenographic, and bacteriological improvement was noted in many cases. There was no clinical intolerance except for the unpleasant odor of the breath.

Because of the unpleasant smell of ethyl mercaptan, it was considered desirable that a therapeutically acceptable compound should release the thiol only after absorption. Further research by Davies et al. (1956) led to the discovery of diethyldithiol-*iso*-phthalate (Etisul). Antituberculosis activity of Etisul in mice and guinea pigs was reported by Davies and Driver (1957), and its suppressive activity in murine leprosy in the cornea of mice was reported by Naguib and Robson (1956). Recently, Etisul has been claimed to be effective in the treatment of leprosy (Davey and Hogerzeil, 1959) but this is contradicted by Dharmendra and Noordeen (1961).

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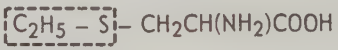
The present report deals with studies of the activity of four ethyl mercaptan compounds in murine leprosy in mice.

## Materials and Methods

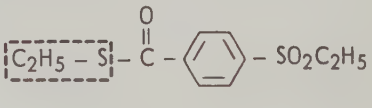
Female albino mice of the general purpose strain of the National Institutes of Health, weighing 16 to 20 g, were used in groups of 10 (for 2-week experiments) or 20 (for 3-month experiments), each group being caged separately. Each mouse was inoculated intraperitoneally with 0.5 ml of a 1:30 suspension in normal saline of the omenta and pelvic fatty pads from mice which had been infected 4 to 5 months previously with the Hawaiian strain of *Mycobacterium lepraemurium*.

Four ethyl mercaptan compounds were used: S-ethyl-L-cysteine, ethylthio-*p*-ethylsulfonyl benzoate, sodium ethylthiosulfate, and Etisul (Fig. 1). All drugs were mixed in the powdered Purina rat chow. Treatment was commenced on the day after inoculation. All animals were sacrificed at the end of 2 weeks or 3 months.

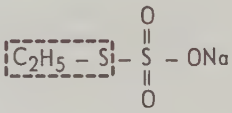
S-Ethyl-*L*-cysteine



Ethylthio-*p*-ethylsulfonyl-benzoate



Sodium ethyl thiosulfate



Diethyldithiol*iso*phthalate or Etisul

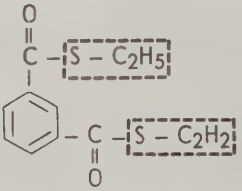


FIG. 1. *Chemical structure of ethyl mercaptan compounds.*

This technique of 3-months chemotherapeutic assay, using intraperitoneally infected mice, has been reported (Chang, 1953). The average weight for the group of the omenta and pelvic fatty pads and a "leprosy index," which is an average evaluation of the gross lesions in various sites and organs, are the basis of the assay. In some

experiments, bacillary counts made from standard suspensions of the spleen and liver were included.

A 3-week chemotherapeutic assay was also employed. In this short test, microscopic grading of lesions of mesenteric spreads and bacillary counts of smears from the spleen and liver are used as the criteria for estimating drug activity. Details of techniques for these studies have been published elsewhere (Chang, 1959). Briefly, the lesions in the mesentery are examined microscopically, using the low-power objective and a 5-mm square grill in the ocular. Lesions are graded from 1 to 10, according to the number of infected cells per square. Bacillary counts for the organs in both 3-week and 3-month experiments are made on smears prepared from suspensions. The total volume of the suspensions is 4 ml for each spleen and 7 ml for each liver. Using a 2-mm platinum loop, duplicate smears, 18 mm in diameter, are made from undiluted or suitably diluted suspensions. The bacillary count for each organ is the average for 200 squares (oil immersion).

## Results

*S-Ethyl-L-cysteine and ethylthio-p-ethylsulfonyl benzoate.* The effect of these two drugs was studied only in a 3-month experiment. S-ethyl-L-cysteine showed no activity; the weights of omenta and pelvic fatty pads and the leprosy index of the treated animals were essentially the same as those of the untreated leprosy controls (Table 1). Ethylthio-*p*-ethylsulfonyl benzoate revealed definite activity; the weights of the omenta and pelvic fatty pads of the treated animals were only one-half to one-third those of the controls. The leprosy index of the treated animals was 6.13, as compared to 10.99 in the controls.

TABLE 1. *The effect of S-ethyl-L-cysteine and ethylthio-p-ethylsulfonyl benzoate in murine leprosy in a 3-month experiment*

| Drug | Dose in diet* | Mice died/used | Avg body wt | Avg wt of omentum | Avg wt of pelvic fatty pads | Avg leprosy index |
|---|---|---|---|---|---|---|
| | % | *no.* | *g* | *g* | *g* | |
| Leprosy control (untreated) | – | 0/20 | 21.5 | 0.12 | 1.15 | 10.99 |
| S-ethyl-L-cysteine | 0.2 | 0/20 | 21.3 | 0.14 | 1.28 | 10.82 |
| Ethylthio-*p*-ethyl-sulfonyl benzoate | 0.2 | 4/20† | 21.6 | 0.05 | 0.57 | 6.13 |

*In this and the following tables, the dose of drugs may be calculated as mg per kg of body weight on the basis of 4 g of powdered food taken by a 20-g mouse per day. Thus, 0.2% in the diet equals about 400 mg/kg, etc.

†Of the four deaths in this group, one each died on the 24th and 38th day and two on the 46th day of the infection. The drug was well tolerated by the rest of the animals of this group. The death of the four animals was probably due to intercurrent diseases.

*Sodium ethylthiosulfate.* The effect of sodium ethylthiosulfate was studied in both 3-week and 3-month experiments. Doses of 0.1, 0.2, and 0.5% in the diet were employed, using 4,4'-diaminodiphenyl sulfone (DDS) (0.1%) as the reference standard for drug activity (Table 2).

TABLE 2. *Effect of sodium ethylthiosulfate in murine leprosy*

| Drug | Dose in diet | Mice died/used | Infected cells in mesenteric spreads | Avg body wt | Avg wt of omentum | Avg wt of pelvic fatty pads | Avg no. of bacilli per oil immersion field | | Avg leprosy index |
|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | Splenic smear | Liver smear | |
| *3-Week experiment* | % | no. | avg score | g | g | g | | | |
| Leprosy control (untreated) | – | 0/10 | 3.0 | | | | 1.2 | 3.0 | |
| DDS | 0.1 | 3/10 | 2.6 | | | | 1.4 | 5.0 | |
| Sodium ethylthiosulfate | 0.1 | 1/10 | 1.1 | | | | 0.4 | 1.5 | |
| Sodium ethylthiosulfate | 0.2 | 0/10 | 0.9 | | | | 0.2 | 0.9 | |
| Sodium ethylthiosulfate | 0.5 | 0/10 | 0.6 | | | | 0.3 | 0.7 | |
| *3-Month experiment* | | | | | | | | | |
| Leprosy control (untreated) | – | 0/20 | | 26.3 | 0.18 | 1.67 | 82 | 543 | 8.68 |
| DDS | 0.1 | 1/20 | | 24.9 | 0.14 | 0.85 | 14 | 83 | 5.55 |
| Sodium ethylthiosulfate | 0.1 | 0/20 | | 25.8 | 0.14 | 1.42 | 7 | 418 | 7.33 |
| Sodium ethylthiosulfate | 0.2 | 0/20 | | 28.1 | 0.10 | 1.07 | 6 | 80 | 4.43 |
| Sodium ethylthiosulfate | 0.5 | 0/20 | | 25.5 | 0.05 | 0.78 | 1 | 2 | 0.43 |

In the 3-week experiment, each of the three doses of sodium ethylthiosulfate showed suppressive activity, as evidenced by fewer infected cells in the mesenteric spreads and lower bacillary counts for the spleen and liver in comparison with the findings in the untreated controls. DDS showed no suppressive activity in this short-term experiment.

In the 3-month experiment, however, DDS revealed definite activity. The weights of omenta and pelvic fatty pads and the bacillary counts of the spleen and liver of the DDS-treated group were lower than those of the controls. The leprosy index of the DDS-treated group was 5.55, and that of the controls was 8.68.

In the highest dosage (0.5% in the diet), the repressive activity of sodium ethylthiosulfate was much more marked than that of DDS. In the medium dose, 0.2%, the results were about as good as with the reference drug, and the lowest dose, 0.1%, showed doubtful activity.

*Etisul.* Three doses of Etisul were used in both the 3-week and 3-month experiments (Table 3). Isoniazid was used as the standard of reference.

In the 3-week experiment, isoniazid (0.01%) and all doses of Etisul (0.1, 0.2, and 0.5%), showed definite activity which was similar in degree one to another.

In the first of two experiments in the 3-month test, isoniazid was used in doses of 0.005 and 0.01% and Etisul in doses of 0.1, 0.2, and 0.5%. Both drugs showed marked suppressive activity in all the doses used. The activity of the smaller dose of isoniazid was similar to that of the lower doses of Etisul, although the activity of the highest doses of both drugs was practically the same.

In the second experiment, the activity of the highest doses of Etisul and of isoniazid was again compared. Bacillary counts of spleen and liver were included. Animals treated with both drugs revealed only minimal lesions, in comparison with the extensive ones observed in the controls.

TABLE 3. *Effect of Etisul in murine leprosy*

| Drug | Dose in diet | Mice died/used | Infected cells in mesenteric spreads | Avg body wt | Avg wt of omentum | Avg wt of pelvic fatty pads | Avg no. of bacilli per oil immersion field | | Avg leprosy index |
|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | Splenic smear | Liver smear | |
| *3-Week experiment* | % | *no.* | *avg score* | *g* | *g* | *g* | | | |
| Leprosy control (untreated) | – | 0/10 | 2.3 | | | | 7.1 | 13.1 | |
| Isoniazid | 0.01 | 0/10 | 0.6 | | | | 0.7 | 2.2 | |
| Etisul | 0.1 | 0/10 | 0.8 | | | | 0.9 | 5.5 | |
| Etisul | 0.2 | 0/10 | 0.7 | | | | 1.8 | 2.7 | |
| Etisul | 0.5 | 0/10 | 0.6 | | | | 2.7 | 3.3 | |
| *3-Month experiment* | | | | | | | | | |
| Leprosy control (untreated) | – | 0/20 | | 25.3 | 0.23 | 1.27 | | | 8.43 |
| Isoniazid | 0.005 | 2/20 | | 26.8 | 0.13 | 0.73 | | | 3.12 |
| Isoniazid | 0.01 | 1/20 | | 28.0 | 0.04 | 0.70 | | | 0.95 |
| Etisul | 0.1 | 0/20 | | 26.1 | 0.16 | 0.82 | | | 4.38 |
| Etisul | 0.2 | 0/20 | | 27.0 | 0.13 | 0.73 | | | 2.48 |
| Etisul | 0.5 | 0/20 | | 26.0 | 0.08 | 0.59 | | | 0.65 |
| *3-Month experiment* | | | | | | | | | |
| Leprosy control (untreated) | – | 1/20 | | 22.6 | 0.27 | 1.12 | 633 | 1386 | 10.75 |
| Isoniazid | 0.01 | 1/20 | | 23.1 | 0.03 | 0.34 | 3 | 32 | 0.69 |
| Etisul | 0.5 | 1/20 | | 25.5 | 0.04 | 0.59 | 1 | 1 | 0.37 |

Sodium ethylthiosulfate, Etisul, and isoniazid, in the highest doses used, showed approximately equivalent repressive activity. A comparison was made of the average survival time of animals in which treatment was continued until death. The average survival time was 118 days for untreated controls (Table 4). For animals treated with the highest dosage of isoniazid, sodium ethylthiosulfate, and Etisul, survival time was 279, 263, and 303 days, respectively. Thus, survival

TABLE 4. *Average survival time of animals continuously treated with sodium ethylthiosulfate and Etisul**

| Drug | Dose in diet | Avg survival time | Available leprosy index† |
|---|---|---|---|
| | % | *days* | |
| Leprosy control (untreated) | – | 118 | 18.07 (14)‡ |
| Isoniazid | 0.01 | 279 | 12.70 (5) |
| Sodium ethylthiosulfate | 0.5 | 263 | 10.08 (6) |
| Etisul | 0.5 | 303 | 11.35 (10) |

*A total of 20 animals were used for each group.

†Figures in parentheses are the number of animals available for the evaluation of leprosy indices.

‡Data of previous experiments (Chang, 1957).

was markedly prolonged in all treated animals. Etisul appeared slightly superior to the other two drugs.

The extent of leprosy growth was evaluated at death for all animals except those which showed marked postmortem changes. Data were available for from 5 to 10 animals in each of the three treated groups. All showed extensive lesions (Table 4). It is concluded, therefore, that these compounds are able to suppress the growth of *M. lepraemurium* for only a limited period.

Additional studies on the acquirement of resistance of *M. lepraemurium* to ethyl mercaptan compounds were finished after this paper was written and provided the following data.

Three sets of animals were inoculated with emulsified lesions obtained from a mouse of the series shown in Table 4. One source mouse had been treated with sodium ethylthiosulfate (0.5%) for 351 days, the second with Etisul (0.5%) for the same length of time, and the third with isoniazid (0.01%) for 407 days. Each set was divided into two groups. The drug and dosage for one group of each set corresponded to those used in the original experiment; the other group of each set was used as a control. Animals were sacrificed 3 months later. Isoniazid revealed no activity, as shown by the similar weights of omenta and pelvic fatty paids and the leprosy index to those of the controls (Table 5). On the other hand, the lesions

TABLE 5. *Drug resistance in murine leprosy. Results of 3-month treatment with isoniazid, sodium ethylthiosulfate, or Etisul of mice inoculated with lesions from animals previously treated with the same drug*

| Drug | Dose in diet | Mice died/used | Body wt | Wt of omentum | Wt of pelvic fatty pads | Leprosy index |
|---|---|---|---|---|---|---|
| | % | *no.* | *g* | *g* | *g* | |
| Leprosy control (untreated) | – | 2/20 | 28.2 | 0.47 | 1.27 | 9.20 |
| Isoniazid | 0.01 | 1/20 | 27.1 | 0.45 | 1.73 | 10.35 |
| Leprosy control (untreated) | – | 1/20 | 27.0 | 0.20 | 1.93 | 9.18 |
| Sodium ethylthiosulfate | 0.5 | 1/20 | 25.3 | 0.08 | 0.74 | 2.79 |
| Leprosy control (untreated) | – | 0/20 | 27.0 | 0.19 | 1.48 | 8.48 |
| Etisul | 0.5 | 0/20 | 25.9 | 0.09 | 0.92 | 2.65 |

of the animals treated with the two ethyl mercaptan compounds were much smaller than those of the controls and the leprosy indices only about one-third as high. Therefore, *M. lepraemurium* is assumed to have been completely resistant to isoniazid at this time, but only slightly resistant to the ethyl mercaptan compounds.

It is concluded that sodium ethylthiosulfate and Etisul possess a repressive activity in murine leprosy approaching that of isoniazid, but that the development of resistance by *M. lepraemurium* to these mercaptans is slower than to isoniazid.

## Discussion

From the above findings, it is evident that certain ethyl mercaptan compounds possess definite activity in the suppression of murine leprosy. Of the four compounds studied, sodium ethylthiosulfate and Etisul showed highest activity. Ethylthio-*p*-ethylsulfonyl benzoate was the next best, while S-ethyl-L-cysteine was inactive. The potency of Etisul was similar, if not superior, to that of sodium ethylthiosulfate. Both compounds showed activity approaching that of isoniazid, which has given the best results for the present. Similar findings with Etisul have been reported by Naguib and Robson (1956) in mice infected with *M. lepraemurium* by the intracorneal route. It should be noted that the comparison of potency was based on the full dose of isoniazid (0.01%); with this dosage, only minimal lesions were observed in the animals.

Rapid development of resistance by *M. lepraemurium* has been found in animals treated with isoniazid (Bushby and Barnett, 1953). It would be of interest to learn if ethyl mercaptan compounds also cause development of resistance. Since marked lesions are observed in animals continuously treated with these drugs, it seems probable that mercaptans also produce resistance in the same pattern as isoniazid. Further studies along this line are in progress. Recent clinical trials have indicated that Etisul exhibits a therapeutic effect only in the first few months of treatment, suggesting that development of resistance also occurs in leprosy.

Etisul is an oily liquid which causes marked irritation of the mucosa and tissues. The only suitable method for administration in humans is by inunction which is troublesome and time consuming. Sodium ethylthiosulfate, on the other hand, can be administered orally. Since it is as potent as Etisul in murine leprosy, a broad scale trial of sodium ethylthiosulfate in leprosy seems justified.

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## Literature Cited

Anderson, H. H., and Y. C. Chin. 1947. Antibiotic activity of subtlin and streptomycin in the presence of BAL. Science **106**:643-644.

Brown, H. D., A. R. Matzuk, H. J. Becker, J. P. Conbere, J. M. Constantin, M. Solotorovsky, S. Winsten, E. Ironson, and J. H. Quastel. 1954. The antituberculosis activity of some ethylmercapto compounds. J. Am. Chem. Soc. **76**:3680.

Bushby, S. R. M., and M. Barnett. 1953. Isoniazid resistance in murine leprosy. Intern. J. Leprosy **21**:467-468.

Chang, Y. T. 1953. Chemotherapy of murine leprosy. I. The use of mouse leprosy as chemotherapeutic test. Intern. J. Leprosy **23**:47-56.

Chang, Y. T. 1957. Chemotherapy of murine leprosy. VI. The effects of isonicotinylhydrazone of 2-carboxymethoxy-3-methoxybenzaldehyde (Compound 373) and isonicotinylhydrazone of 2-carboxymethoxybenzaldehyde (Compound 377) on mouse leprosy. Intern. J. Leprosy **25**: 130-146.

Chang, Y. T. 1959. Evolution of murine leprosy. Am. Rev. Tuberc. Pulmonary Diseases **79**:805-809.

Davey, R. F., and L. M. Hogerzeil. 1959. Diethyldithiol*iso*phthalate in the treatment of leprosy (ETIP or "Etisul"): A progress report. Leprosy Rev. **30**:61-72.

Davies, G. E., and G. W. Driver. 1957. The antituberculous activity of ethylthiolester, with particular reference to diethyldithiol*iso*phthalate Brit. J. Pharmacol. **12**:434-437.

Davies, G. E., G. W. Driver, E. Hoggarth, A. R. Martin, M. F. C. Paige, F. L. Rose, and B. R. Wilson. 1956. Studies in the chemotherapy of tuberculosis: Ethyl mercaptan and related compounds. Brit. J. Pharmacol. **11**:351-356.

Dharmendra, and S. K. Noordeen. 1961. Diethyldithiol*iso*phthalate (Etisul) in the treatment of leprosy. Intern. J. Leprosy **29**:34-45.

Mathewson, J. A., M. Thoren, and H. C. Hinshaw. 1956. A clinical trial of S-ethyl-*L*-cysteine in pulmonary tuberculosis. Am. Rev. Tuberc. Pulmonary Diseases **74**:142-144.

Naguib, M., and J. M. Robson. 1956. The activity of diethyldithiol*iso*phthalate alone and combined with isoniazid in the treatment of murine leprosy in the mouse cornea. Lancet **1**:411-412.

del Pianto, E. 1950. Chemioterapia della tubercolosi con il 2-mercaptobenzotiazolo e suoi deraviti in associazione con i sali die S-esteri dell'acido tiosolforico. Ricerca sci. **20**:83-101.

del Pianto, E. 1953. Activite antituberculare dell'etiltiosolfato di sodio. Ricerca sci. **23**:1785-1793.

del Pianto, E. 1958. The thioethyl-compounds in therapy of leprosy. I. Clinical results in a pilot trial with sodium ethyl thiosulfate. Trans. 6th Intern. Cong. of Leprosy. p. 279-291.

Solotorovsky, M., E. Ironson, and S. Winten. 1956. The antituberculous activity of ethyl mercaptan. Am. Rev. Tuberc. Pulmonary Diseases **74**:72-77.

# EFFECT OF $N^1$, $N^1$-ANHYDROBIS-($\beta$-HYDROXYETHYL) BIGUANIDE-HYDROCHLORIDE ON ASIAN INFLUENZA VIRUS IN VOLUNTEERS

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Interest in the chemotherapy of viral infections has recently been supported by increased activity and research on antiviral agents. As an outgrowth of this interest, the need for meaningful methods of testing the antiviral activity of potentially useful substances has assumed increased importance. Compounds that inactivate viruses upon contact can easily be recognized and tested. Other substances that inhibit virus multiplication can sometimes be studied in tissue cultures or small laboratory animals. Often, the materials are too toxic or pharmacologically inappropriate for use in man, which is the aim of most of the interest in antiviral chemotherapy. But even if apparently useful drugs can be given safely and easily to man, evaluation of their effectiveness is complex.

Studies on antiviral chemotherapy usually have been in the nature of field studies in which a compound is administered therapeutically or prophylactically to a large test population. The incidence and severity of clinical illness observed in the test group are often the only basis for evaluation. More reliable measures require extensive and specialized laboratory support. In the absence of an epidemic viral infection, clinical indices are subject to so many variables as to make them virtually useless measures of evaluation. At the time of epidemic viral infection, conditions are seldom prepared for the proper and careful study of a chemotherapeutic drug of unknown effectiveness.

Studies in volunteers provide many advantages that may be applied to the study of antiviral drugs. Variable host factors can be matched in the test groups, the treatment regimen can be prescribed, and the specificity, time, and severity of the infectious challenge in

relation to treatment can be well controlled. In the studies reported here, observations that have been found useful in the study of viral respiratory infections in volunteers (Jackson et al., 1958) have been explored as a means of obtaining controlled data from a structured experiment designed to measure the antiviral activity of a study drug, $N^1,N^1$-anhydrobis-($\beta$-hydroxyethyl) biguanide-hydrochloride (ABOB), against Asian influenza virus infection.

## Materials and Methods

*Challenge virus.* A seed culture of the attenuated Asian influenza virus strain used as a live virus vaccine in Russia and elsewhere (Zhdanov and Soloviev, 1960; Meiklejohn, 1960), designated A2-134 and suspended in allantoic fluid, was obtained through the Commission on Influenza, Armed Forces Epidemiological Board. The virus was subpassaged once in the allantoic cavity of chicken embryos and the fluid harvested aseptically, clarified free from cells, and shown to contain no contaminating bacteria. The fluid harvest had an egg-infectious dose for 50% of eggs ($EID_{50}$) of $10^{6.7}$ and a hemagglutination titer for human group 0 erythrocytes of 1:256. The harvest was frozen at -70 C, tested for infectiousness in a few subjects, and stored until used for the challenge of volunteers as noted below. After completion of the experimental challenge the material given to volunteers was shown to have retained 1,000 $EID_{50}$ of influenza virus per 0.1 ml.

A sample of the infected allantoic fluid was heat-inactivated at 62 C for 15 min before its administration to a group of 10 volunteers. This material contained $10^{2.4}$ $EID_{50}$ and had a hemagglutination titer for human group 0 erythrocytes of 1:256.

*Study drug.* ABOB was prepared in sustained-release tablets containing 350 mg each. These were color-coded into two allotments. Identical placebo tablets were prepared and given two different color codes. These were administered to volunteers as noted below.

*Volunteers.* Male college students above 21 years of age, and female students above 18 years of age, were permitted to participate. A total of 330 subjects completed the initial phase of the study, and 183 subjects with a serum antibody titer of 10 or less and 22 with a titer greater than 10 were included in the final test of the drug.

*Reisolation of virus.* Secretion from the middle nasal meatus and inferior turbinates was collected with cotton swabs which were thoroughly rinsed in 1.5 ml of Hanks buffered saline solution, sealed, and immediately frozen at -76 C. Subsequently, allantoic inoculations of 0.2 ml into each of three 11-day-old chicken embryos were made. The allantoic fluid from each set of eggs was harvested and pooled after 48 hr, tested for virus by hemagglutination, and 0.5 ml inoculated directly into a second set of three chicken embryos. All

virus isolates were identified as influenza $A_2$ by specific neutralization tests.

*Nasal smears.* Nasal epithelial cells were collected from the inferior turbinate with a curette and smeared on slides, which were then acetone-fixed and dried. Examination for cytotoxicity was performed after staining with Papanicalau stain. Detection of virus by means of the indirect and direct method of fluorescent antibody was made by dividing two equal areas on the slide. To one half was added specific anti-influenza guinea pig serum and to the other, normal guinea pig serum. The slides were incubated at 37 C for 45 min and washed. When the indirect method was used, fluorescein-conjugated rabbit anti-guinea pig globulin rabbit serum was added to the entire slide, which was reincubated and washed. In the direct technique, fluorescein-conjugated anti-influenza guinea pig serum was used. Detection of microscopic specific fluorescence was made using a dark-field condenser system and appropriate filters.

*Serology.* The influenza antibody titer was determined by the hemagglutination-inhibition technique using serial twofold dilutions of serum, after treatment with 0.01M periodate for removal of nonspecific inhibitor. Chicken cell erythrocytes and 4 HA units of influenza virus, strain A/JAP/305-57, were added to each tube. Hemagglutination was read after 1 hr at 4 C. All initial serum specimens were rerun simultaneously with convalescent specimens obtained after the experimental virus challenge. Only rises of fourfold or greater magnitude were considered significant.

*Antiviral test.* The pretest structure of the experiment with volunteers was as follows: (i) determination of specific antibody status and the formation of matched groups; (ii) pretreatment with a high dosage of ABOB or placebo using the double-blind method; and (iii) challenge of volunteers with attenuated Asian flu virus or uninfected allantoic fluid, again using the double-blind method.

Drug activity was determined by the double-blind assessment from the following: (i) daily record of clinical symptoms; (ii) recovery of virus from nasal swabs taken 2, 4, and 6 days after challenge; (iii) cytotoxicity in exfoliated nasal epithelial cells; (iv) viral replication in the nasal mucosa shown by specific fluorescent antiserum; and (v) frequency, rate, and height of serological antibody response.

A serum specimen was obtained from all potential volunteers approximately 3 weeks before the administration of virus. After determination of the serum anti-influenza antibody titer, the subjects were assigned to one of four matched groups.

Treatment with ABOB was given daily in two 350-mg tablets, in the morning upon arising and at supper time, beginning 24 hr before challenge with the infectious virus and continuing for 5 days following the challenge. This was calculated to give sustained absorption of drug over the 24-hr period in a dose of approximately 20 mg/kg per day.

The influenza virus was administered as nose drops of undiluted allantoic fluid, using 0.2 ml per nares. The subjects reported their symptoms 2 days after the challenge and every other day for a week. A list of respiratory and constitutional symptoms was graded according to severity for each subject on each day. Nasal swabs were obtained before challenge and on the second, fourth, and sixth days after challenge. On the fourth day after challenge a curettement was used to prepare a smear of nasal cells. Convalescent serum specimens were obtained 2, 4, and 6 weeks after the challenge.

## Results

*Clinical illness.* Clinical illness was judged by criteria previously established in volunteer experiments with respiratory viruses (Jackson et al., 1958). The subjects were arranged in groups according to the type of challenge and type of treatment given (Table 1).

TABLE 1. *Clinical illness after the administration of attenuated Asian influenza virus*

| Group and treatment | Subjects | Clinical respiratory illness* | | Symptom score† |
|---|---|---|---|---|
| | *no.* | *no.* | % | |
| Uninfected | | | | |
| Placebo | 20 | 2 | 10 | 3.3 |
| ABOB | 19 | 2 | 10 | 8.0 |
| Virus | | | | |
| Placebo | 80 | 14 | 18 | 7.2 |
| ABOB | 86 | 12 | 14 | 7.7 |

*Established according to predetermined criteria, based on a symptom score, nasal discharge, and volunteer response.

†Mean total symptom score for all members of the group, determined from the daily record of eight symptoms.

Among treated and nontreated volunteers, a 10% incidence of clinical respiratory illness developed in the week after challenge with uninfected allantoic fluid. The attenuated virus caused an appreciable but statistically insignificant increase in clinical respiratory illnesses. The groups are also compared, for the week after challenge, according to the average symptom score, which is an index of the over-all severity of symptoms in the group. The uninfected allantoic fluid produced a few symptoms among the placebo-treated group. The higher value observed among uninfected subjects given ABOB suggests some drug toxicity. Among subjects challenged with virus, no

significant difference was observed with regard to treatment, either in the frequency of clinical respiratory infections or the mean symptom score for the groups.

*Virus reisolation from nasal swabs.* Among subjects given influenza virus, reisolation was made in the first egg passage from nasal swabs taken on day 2 from 23 subjects (30%) who received placebo and 18 (21%) who were given ABOB (Table 2). This difference, suggesting suppression of virus by ABOB treatment, is not statistically significant. Virus was recovered only after a second serial egg passage, indicating specimens that may have had a smaller amount of virus, in 10 and 15% of placebo- and ABOB-treated subjects, respectively. On day 4, no virus isolates were recovered upon the first egg passage. Virus was recovered from one and three subjects in the placebo- and ABOB-treated groups, respectively.

TABLE 2. *Reisolation of Asian influenza virus from nasal swab specimens*

| Days after virus was given | Treatment | Number of specimens | Virus isolates | | | |
|---|---|---|---|---|---|---|
| | | | First egg passage | | Second egg passage | |
| | | | *no.* | % | *no.* | % |
| 2 | Placebo | 77 | 23 | 30 | 8 | 10 |
| 2 | ABOB | 84 | 18 | 21 | 13 | 16 |
| 4 | Placebo | 80 | 0 | – | 1 | 1 |
| 4 | ABOB | 84 | 0 | – | 3 | 4 |

*Cytotoxicity in nasal epithelial cells.* Cytotoxicity of nasal epithelial cells (Fig. 1) was observed in previous experiments in which

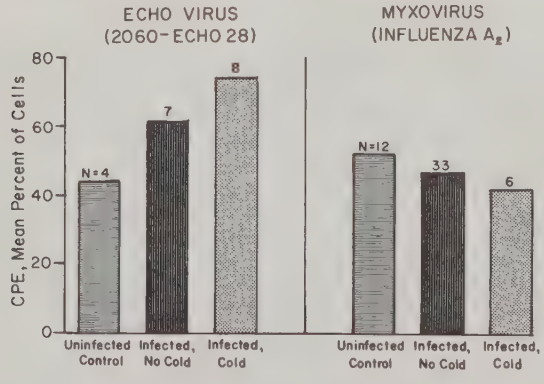


FIG. 1. *Cytopathogenic effect of virus infection upon nasal epithelial cells, 4 days after challenge.*

the infectious agent was an ECHO virus (Jackson et al., 1960). No cytotoxicity in the day 4 smears was observed, however, by the administration of attenuated Asian influenza virus (Fig. 1). This parameter, therefore, offered no criterion for the test of antiviral activity by ABOB.

*Fluorescent staining of influenza antigen in nasal smears.* Fluorescent material in the nasal smears taken 4 days after the experimental challenge was found after staining with conjugated specific anti-Asian influenza serum (Table 3). Among volunteers who received buffer and had no demonstrable contact with the virus or serologic rise to it following the experiment, 2 of 13 smears (15%) were read as positive. Among volunteers who were infected as manifest by a rise in serum antibody after virus administration, 11 of 26 smears, or 42% of subjects treated with ABOB, had fluorescent stainable material, and 8 of 26, or 31% of subjects given placebo, had stainable influenza antigen. The difference between the buffer-negative volunteers and the infected positive volunteers is of borderline statistical significance, whereas, the difference between the ABOB- and placebo-treated groups is insignificant.

TABLE 3. *Influenza viral antigen in nasal smears detected by fluorescent, anti-influenzal antibody*

| Group | Smears | Influenza positive | |
|---|---|---|---|
| | *no.* | *no.* | *%* |
| Uninfected | | | |
| Placebo | 7 | 1 | 14 |
| ABOB | 6 | 1 | 16 |
| Infected | | | |
| Placebo | 26 | 8 | 31 |
| ABOB | 26 | 11 | 42 |

*Serologic antibody response.* The data on the serologic rise in hemagglutination inhibition antibody for Asian influenza are shown in Table 4. Among 39 volunteers who were uninfected, only one four-fold rise in antibody occurred. Among volunteers who had no significant antecedent antibody, serologic evidence of infection was observed in 57 and 53% of subjects treated with placebo or ABOB, respectively. Among subjects who had an initial antibody titer greater than 10, serologic evidence of infection occurred in 20 and 25% of those who received placebo and ABOB, respectively. The mean serum titer and the time of appearance of antibody in the specimens collected at 2, 4, and 6 weeks after the virus challenge were comparable for the placebo- and ABOB-treated groups.

Heat-inactivated virus given to 10 volunteers elicited an antibody response in only one of them. Also, infection occurred more than twice as often among subjects without antibody as in the group with

TABLE 4. *Serological antibody response to attenuated asian influenza virus*

| Group | Initial titer | Number | Fourfold rise |
|---|---|---|---|
| | | | % |
| Uninfected | Any | 39 | 3 |
| Virus | | | |
| Placebo | 10< | 70 | 57 |
| ABOB | 10< | 78 | 53 |
| Placebo | >10 | 10 | 20 |
| ABOB | >10 | 8 | 25 |

antecedent antibody. Thus, the results suggest that the observed serologic data were not obtained because of the booster effect from a small amount of influenza viral antigen, but rather as the result of infection and in vivo poliferation of viral antigens.

Reisolation of virus was not always accompanied by a significant antibody response. Four of 30 subjects treated with ABOB and four of 21 subjects given placebo had virus isolated on the first egg passage of their specimen collected on the second day after challenge with no rise in antibody titer. Virus was reisolated from four of 14 persons receiving ABOB and four of 16 persons receiving placebo who had a twofold rise in antibody. Among subjects who had a fourfold or greater rise, virus was recovered from 10 to 42 on treatment with ABOB and 15 of 43 treated with placebo.

## Discussion

Several previous studies have shown both a favorable and unfavorable effect of ABOB treatment and prevention of influenza (Melander, 1960; Sjoberg, 1960; Alderman et al., 1960; Watson, 1960). The use of volunteers permitted the experiment to be structured and controlled so that the anti-influenza activity of ABOB could be compared against placebo treatment in a double-blind protocol. The parameters selected for measuring efficacy permitted collection of data suitable for interpretation in four of the five indices used.

Although the amount of illness produced by the attenuated influenza virus was small, no significant decrease in illness or symptoms was demonstrated, and, on the other hand, the drug itself may have caused some symptomatic side effects. One might argue that the difference between 14 and 18% in clinical respiratory illness represents a 50% reduction in the excess illness over that observed in the uninfected controls. Such magnification of a statistically insignificant difference does not seem warranted, but it must be granted that a less attenuated virus might have made the difference greater.

A similar situation pertains to the reisolation of virus from nasal secretions on the first egg passage. The reduction is statistically insignificant and essentially nil if second passage isolates also are included. Since the latter, however, may represent some suppression in the quantity of virus in the specimen, which might be clinically beneficial, it cannot be entirely ignored. Considering only the difference in the frequency of first-passage isolates, ABOB treatment decreased virus recovery approximately 30%.

Cytotoxicity of the nasal epithelial cells may be related to the type of virus used for infection. The cytopathogenic effect of ECHO viruses and the lack of cytopathogenic effect of myxoviruses in tissue cultures is similar to that observed in the nasal epithelium of our volunteers.

Autofluorescence and nonspecific staining with fluorescent-conjugated anti-influenzal serum caused some problem in the double-blind interpretation of nasal smears. However, the procedure gave useful comparative information and showed no advantage resulting from ABOB treatment. Retrospectively, the detection of antigen would have been more useful if done earlier in the course of infection.

Serological studies were the most definitive of all the tests made and were conclusively negative with regard to any beneficial effect from treatment with ABOB. It is possible, however, that the host response and the serological technique of determining titer by two-fold dilutions are not sufficiently sensitive to discriminate suppression of virus growth. On the other hand, effective therapy of other infections and certainly prophylactic treatment has been shown by less antibody response. The experiment in volunteers was designed to give ABOB the advantage of pretreatment and relatively high sustained dosage. Under these conditions, treatment failed to prevent infection with an attenuated influenza virus, and the statistically insignificant suggestion of slight suppression of influenza virus must be considered as offering little expectation of clinical benefit from use of the drug for the treatment or prevention of influenza.

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## Literature Cited

Alderman, E. R. et al. 1960. Trial of an anti-viral agent. Practitioner **185**:795-797.

Jackson, G. G., H. F. Dowling, I. G. Spiesman, and A. V. Boand. 1958. Transmission of the common cold to volunteers under controlled conditions. I. The common cold as a clinical entity. A. M. A. Arch. Internal Med. **101**:267-278.

Jackson, G. G., H. F. Dowling, T. O. Anderson, L. Riff, J. Saporta, and M. Turck. 1960. Susceptibility and immunity to common upper respiratory viral infections - the common cold. Ann. Internal Med. **53**:719-738.

Melander, B. 1960. $N^1$, $N^1$-anhydrobis-(beta-hydroxyethyl) biguanidehydrochloride (ABOB) in prophylaxis and suppression of experimental influenza. Antibiotics & Chemotherapy **10**:34-45.

Meiklejohn, G. 1960. Observations on live influenza vaccine. J. Am. Med. Assoc. **172**:1354-1356.

Sjoberg, B. 1960. Experiments on prophylaxis and suppression of epidemic influenza with $N^1$, $N^1$-anhydrobis-(beta-hydroxyethyl) biguanide-hydrochloride (ABOB). A double blind study. Antibiotic Med. & Clin. Therapy **7**:97-102.

Watson, G. I. 1960. "Virugon" ("Flumidin"): no benefit in two respiratory diseases. Brit. Med. J. **2**:1785-1786.

Zhdanov, V. M., and V. D. Soloviev. 1960. Some results of the study of Asian influenza. International Conference on Asian Influenza, p. 178-187.

# NONSPECIFIC PROTECTION AGAINST NEUROTROPIC INFLUENZA

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Attempts to enhance host resistance to gram-negative bacteria and endotoxins derived from them have been made under both clinical and experimental conditions (Culver, 1917; Moore, 1944). The use of chicken embryos has been limited in studying this phenomenon because of the known lethal effect of certain endotoxins on them (Smith and Thomas, 1956). However, certain strains of *Escherichia coli* actively grow in the allantois of the embryo without killing it (Finkelstein and Ranson, 1960). An opportunity was thereby afforded to study what modification of the effects of neurotropic influenza virus an allantoic infection with *E. coli* could induce.

Given intravenously to chicken embryos, neurotropic influenza (NWS) virus, a variant of the WS strain of influenza type A, produces death and characteristic hemorrhages in brain as well as in other tissues. Rupture of small blood vessels is preceded by swelling of endothelial cells and dilation and engorgement of capillaries (Hook and Wagner, 1957*b*) where viral antigen appears to be localized (Hook et al., 1960). Allantoic infection with homologous or heterologous myxoviruses has recently been demonstrated to limit mortality and reduce the hemorrhagic manifestations of this virus in chicken embryos (Grossberg, Hook, and Wagner, 1962). The present studies indicate that allantoic infection by a strain of *E. coli* avirulent for the embryo can modify the effects of neurotropic influenza virus given intravenously.

## Materials and Methods

*Viruses.* NWS strain of influenza type A virus, the "neurotropic" variant (Stuart-Harris, 1939) of the WS parent strain, was used in 43rd to 46th chicken embryo passage. Seed virus, as infected allantoic fluid, was prepared as harvest from 12-day-old embryos inoculated intra-allantoically 48 hr before with 0.1 ml of $10^{-3}$ dilution of stock virus. Western equine encephalitis (WEE) virus had been originally isolated by K. F. Meyer and maintained in mouse brain; the

passage history of the strain used in this laboratory has been described (Grossberg and Holland, *in press*). WEE virus was used as 10% suckling mouse brain in 0.1% bovine albumin in Hanks' balanced salt solution (BSS) with penicillin and streptomycin. Viruses were stored in glass ampoules in a dry-ice chest. Dilutions of virus were made in 0.1% bovine albumin in BSS with penicillin, 100 units, and streptomycin, 100 μg/ml.

*Bacteria. E. coli,* serotype 0111:B4:2, was kindly sent by R. A. Finkelstein of Walter Reed Army Institute of Research (WRAIR) and had been designated by him as "strain no. 9" (Finkelstein, 1961), after his receipt of it from Viola M. Young, WRAIR, who had in turn received it from W. H. Ewing of the Enteric Disease Unit at Chamblee, Ga., in 1954 (Young, *personal communication*). At present the strain gives typical biochemical reactions of *E. coli* but does not produce the usual metallic sheen on eosin-methylene blue agar medium. It is sensitive to streptomycin (200 μg/ml) and neomycin (12.5 μg/ml) and resistant to penicillin (100 units/ml).

Unless otherwise indicated, samples from a single 18-hr culture in Trypticase soy broth which had been glass ampouled and kept at -70 C were used after thawing immediately prior to use. Viability counts on this frozen "seed" by the usual nutrient agar pour plate method using decimal dilutions in nutrient broth showed no decline over a 10-month period from initial titers of $5 \times 10^8$/ml.

*Chicken embryos.* Fertile eggs from White Leghorn chickens were incubated at 38 C prior to experimental use; after inoculation embryos were kept at 36 C. Embryos were inoculated with about 300 egg infectivity ($EID_{50}$) doses of NWS virus intravenously at 14 days of age through a small window over a chorioallantoic vein, and with bacteria (or sterile nutrient broth) intra-allantoically by methods previously outlined (Hook and Wagner, 1957*a*). Inoculated embryos were candled daily to observe viability; dead embryos were examined grossly for presence and extent of brain hemorrhage for 96 hr after virus challenge. Hemorrhages were graded on a scale of 0 to 3+ and expressed as a percentage of total possible brain lesions (Hook and Wagner, 1957*a*). Embryos dying within 24 hr after intravenous challenge were excluded from computation since mortality from NWS virus begins after this period. The $EID_{50}$ of virus was determined by allantoic subinoculation of 0.1 ml in 10-day-old chicken embryos (five embryos per dilution) with subsequent assay of allantoic fluids for the presence of hemagglutinins tested with equal volumes of a 1% suspension of fowl erythrocytes in normal saline.

## Results

*Protection by prior allantoic infection with E. coli.* Living *E. coli* inoculated into the allantois of chicken embryos inhibited the

appearance of hemorrhagic manifestations caused by intravenous inoculation of NWS virus. Table 1 summarizes an experiment in which brain hemorrhages caused by NWS virus were remarkably reduced in embryos pretreated with *E. coli* allantoically 24 hr prior to intravenous NWS virus challenge; however, no effect upon the mortality pattern was observable. Despite the multiplication of *E. coli* ($10^5$ organisms in inoculum to maximal growth of $10^8$/ml), no organisms were cultured from brain for more than 84 hr and only occasional deaths (about 10%) from *E. coli* alone were noted after 100 hr after allantoic inoculation. Similar protection has been obtained with other *E. coli* strains avirulent for chicken embryos.

Pooled allantoic fluids harvested from embryos previously inoculated with living *E. coli* were centrifuged at 100,000 × *g* to remove most but not all *E. coli* and treated with neomycin, 50 μg/ml. When 0.2 ml of this material was inoculated intra-allantoically at 24 hr and again 4 hr before intravenous challenge with NWS virus, diminution in brain lesions and slight prolongation of survival were noted as compared to controls pre-inoculated with normal allantoic fluid. Prior allantoic *E. coli* infection even with a 48-hr interval to intravenous challenge with WEE virus failed to protect embryos against the rapid killing effect of this virus.

*Relation of time of E. coli allantoic infection to protection.* A somewhat greater effect was achieved against hemorrhagic encephalopathy when the interval between initiation of allantoic infection was prolonged from 24 hr to 48 hr prior to intravenous virus challenge (Table 1). Exaltation of virus effect was achieved when interval to challenge was reduced to 12 hr or less, as manifested by an increase in mortality and brain hemorrhages.

TABLE 1. *Resistance to intravenous challenge with 300 $EID_{50}$ of NWS virus in 14-day-old chicken embryos previously infected allantoically with living Escherichia coli*

| Allantoic inoculum | Interval to iv challenge | Cumulative brain lesion score* | | |
|---|---|---|---|---|
| | | 48 hr | 72 hr | 96 hr |
| | *hr* | % | % | % |
| *E. coli* | 24 | 22 | 30 | – |
| Sterile broth | 24 | 73 | 81 | – |
| *E. coli* | 48 | 6 | 10 | 10 |
| Sterile broth | 48 | 28 | 67 | 72 |

*After injection of NWS virus intravenously.

*Protective effect by killed E. coli.* Living *E. coli* in the allantoic inoculum was not a requirement for the establishment of protection to virus challenge. When a Trypticase soy broth culture of *E. coli* was heated at 70 C for 1 hr so as to destroy viability and inoculated into the allantois of embryos 24 hr prior to intravenous

NWS challenge, protection was achieved against brain lesions comparable to that observed when the inoculum contained living *E. coli* (Table 2). Although it appeared that endotoxin might induce protection, numerous attempts failed to provoke reproducible resistance with a commercially available purified endotoxin from *Salmonella typhosa*.

TABLE 2. *Resistance to iv challenge with NWS virus in chicken embryos given living or dead Escherichia coli allantoically 24 hr previously*

| Allantoic inoculum | Cumulative brain lesion score* | |
|---|---|---|
| | at 48 hr | at 72 hr |
| | % | % |
| Living *E. coli* | 34 | 45 |
| Heat-killed *E. coli*† | 33 | 33 |
| Sterile broth | 75 | 80 |

*After injection of NWS virus intravenously.
†Heated 70 C for 60 min.

*Inhibition of viral multiplication in brain.* A preceding allantoic infection with living *E. coli* appeared to inhibit the multiplication of challenge virus in brain. For this experiment a group of 12- or 13-day-old embryos were inoculated with a $10^{-3}$ dilution of *E. coli* "seed" in 0.1-ml amounts intra-allantoically; control embryos were inoculated with sterile nutrient broth allantoically. At 24 or 48 hr after incubation at 36 C the groups of embryos were challenged intravenously with 300 $EID_{50}$ of NWS virus. Embryos were observed for viability 24 hr after intravenous inoculation and any embryos dead at this time were discarded. All dead embryos were examined for brain hemorrhages 48 hr after intravenous challenge. Prior to aseptic removal of cranial contents to make 10% brain suspensions in 0.1% albumin in BSS, the external surface of each embryo head was treated as follows: (i) gentle washing with copious amounts of running tap water, (ii) application, by pouring, of approximately 50 ml of ethyl ether, followed by (iii) further washing with >200 ml of sterile normal saline. Embryos protected by prior *E. coli* allantoic infection (and exhibiting no brain lesions) had significantly reduced titers of NWS virus compared with titers in control embryo brains where hemorrhages were very intense (3+). Thus, it appeared (Table 3) that the repression of brain hemorrhages in protected embryos could be correlated with inhibition of viral multiplication in brain.

## Discussion

The use of strains of *E. coli* relatively avirulent for the chicken embryo has made it possible to extend observations on nonspecific

TABLE 3. *Inhibition of NWS virus multiplication in brains of chicken embryos protected by an Escherichia coli allantoic infection prior to NWS challenge**

| Interval to challenge | Geometric mean titer of NWS virus ($Log_{10}$ $EID_{50}$/0.1 ml brain) | |
|---|---|---|
| | *E. coli*-protected embryos | Control embryos |
| *hr* | | |
| 24 | 5.5 | 6.8 |
| 48 | 5.8 | 7.5 |

*300 $EID_{50}$ NWS virus given intravenously.

resistance to an experimental host which has no mechanisms for antibody production (Trnka and Riba, 1959) or hypersensitivity (Simonsen, 1957). Reduced susceptibility of the chicken embryo to *Vibrio comma* (Finkelstein and Ranson, 1960) and Newcastle disease virus (Finkelstein, 1961) infection has been clearly demonstrated when these agents were inoculated into the allantois after a preparatory inoculum of *E. coli* or its products into the same organ. The observations of Hook and Wagner (1959 *a*,*b*) have emphasized the importance of local tissue factors in determining the protective effects of endotoxin against *S. typhosa* and the neurotoxic action of influenza virus in mice. The present studies using intravenous virus challenge suggest that resistance to brain damage is enhanced after *E. coli* allantoic infection and not necessarily dependent on local factors in the allantois. They extend the observations of Finkelstein (1961) and demonstrate, moreover, in two parameters that infection in the allantois has some effect within the embryo itself, specifically in brain, where not only hemorrhagic manifestations of NWS virus were reduced but also viral multiplication inhibited.

The reason why embryos die despite the fact that brain lesions are diminished and viral growth inhibited is unclear. Present studies represent the first demonstration where a dissociation could be made in this particular host-virus system between lethality and the appearance of brain hemorrhages. Synergistic effect by the growth of *E. coli* and multiplying NWS virus cannot explain why the same dissociative pattern is observed when the allantoic inoculum contains heat-killed *E. coli*. The partial protective effect observed by transfer of allantoic fluid from embryos inoculated with living *E. coli* to the allantois of otherwise susceptible embryos suggests that either a factor is produced in allantoic fluid which circulates to protect embryo brain or toxic products of *E. coli* affect cells locally as well as within the embryo to impair viral multiplication.

It is of interest that both exaltation and resistance to ectromelia virus in mice given endotoxin appeared to be related to some effect on the reticulo-endothelial system (Gledhill, 1961). It is unlikely that

properdin plays a role in the *E. coli* versus NWS virus system described here since strains of influenza virus are unaffected by properdin (Briody, Cassel, and Medill, 1955). No satisfactory explanation of the mechanisms involved in nonspecific host resistance has thus far been offered.

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### Literature Cited

Briody, B. A., W. A. Cassel, and M. A. Medill. 1955. Adaptation of influenza virus to mice. III. Development of resistance to $\beta$ inhibitor. J. Immunol. **74**:41-45.

Culver, H. 1917. The treatment of gonorrheal infections by the intravenous injection of killed gonococci, meningococci and colon bacilli. J. Am. Med. Assoc. **68**:362-366.

Finkelstein, R. A., and J. P. Ranson. 1960. Nonspecific resistance to experimental cholera in embryonated eggs. J. Exptl. Med. **112**:315-328.

Finkelstein, R. A. 1961. Alteration of susceptibility of embryonated eggs to Newcastle disease virus by *Escherichia coli* and endotoxin. Proc. Soc. Exptl. Biol. Med. **106**:41-44.

Gledhill, A. W. 1961. The effect of bacterial endotoxin on resistance of mice to ectromelia. Brit. J. Exptl. Pathol. **40**:195-202.

Grossberg, S. E., E. W. Hook, and R. R. Wagner. 1962. Hemorrhagic encephalopathy in chicken embryos infected with influenza virus. III. Viral interference at a distant site induced by prior allantoic infection. J. Immunol. **88**:1-8.

Hook, E. W., and R. R. Wagner. 1957*b*. Hemorrhagic encephalopathy in chicken embryos infected with influenza virus. II. Pathology. Bull. Johns Hopkins Hosp. **103**:140-150.

Hook, E. W., and R. R. Wagner. 1959*a*. The resistance-promoting activity of endotoxins and other microbial products. I. Their effect on *Salmonella typhosa* infections of mice. J. Immunol. **83**:302-309.

Hook, E. W., and R. R. Wagner. 1959*b*. The resistance-promoting activity of endotoxins and other microbial products. II. Protection against the neurotoxic action of influenza virus. J. Immunol. **83**:310-317.

Hook, E. W., R. R. Wagner, C. N. Luttrell, and K. Slaten. 1960. Viral infection of vascular endothelium. Clin. Research **8**:31.

Moore, J. E. 1944. The modern treatment of syphillis, 2nd ed. Charles C Thomas, Springfield, Ill.

Simonsen, M. 1957. The impact on the developing embryo and newborn animal of adult homologous cells. Acta Pathol. Microbiol. Scand. **40**:480-500.

Smith, R. T., and L. Thomas. 1956. The lethal effect of endotoxins on the chick embryo. J. Exptl. Med. **104**:217-231.

Stuart-Harris, C. H. 1939. A neurotropic strain of human influenza virus. Lancet **1**:497-499.

Trnka, Z., and I. Riba. 1959. Antibody formation by isolated spleen cells transferred to recipients in absence of homotransplantation reaction. Nature **183**:546-547.

# SUSCEPTIBILITY OF NOCARDIAE AND MYCOBACTERIA TO ACTINOPHAGE

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Lysis of bacteria by bacteriophages is one means of identifying closely related organisms (Felix, 1956). Similarly, sensitivity to actinophage can be used as an index of relationships among actinomycetes (Welsch, Corbaz, and Ettlinger, 1957). Most actinophages are able to attack numerous species within a genus (Alexander and McCoy, 1956), and some actinophages are virulent for members of both the genus *Streptomyces* and the genus *Nocardia* (Bradley and Anderson, 1958; Gilmour, Noller, and Watkins, 1959). On the basis of overlapping susceptibility to actinophages, *Nocardia corallina*, *Mycobacterium rhodochrous*, and *Jensenia canicruria* have been linked closely to *N. brasiliensis* and remotely to the streptomycetes. By this criterion, *Streptoverticillium* is intimately related to *Streptomyces*. Accordingly, a phylogenetic scheme for the actinomycetes, with the verticillates at one extreme and the soft nocardiae at the other, has been proposed (Jones and Bradley, *in press*). In the present study, sensitivity to actinophage has been used to demonstrate interrelationships among various nocardiae and mycobacteria.

## Methods

The actinomycetes, actinophages, and methods of actinophage assay used here have been described previously (Anderson, 1961). Also, methods for isolating and propagating actinophages have been given in detail elsewhere (Bradley, Anderson, and Jones, 1961).

## Results

Diverse strains of *Nocardia* and *Mycobacterium* and a single strain of *Jensenia* were tested for susceptibility to 18 phages isolated on streptomycetes and 5 phages isolated on strains of *Streptoverticillium* (Tables 1 and 2). Only *Nocardia* sp. 52 was sensitive to

streptomycete phages. This organism differed from most strains of *Nocardia* tested in that it consistently produced abundant aerial hyphae with straight spore chains, thereby morphologically resembling the streptomycetes.

TABLE 1. *Susceptibilities of nocardiae, mycobacteria, and other actinomycetes to actinophages*

| Host | Phage | | | | |
|---|---|---|---|---|---|
| | MSP1[a] | WSP1[b] | WSP2[c] | WSP3[d] | MSP21[e] |
| *S. griseus* S86 | 10.3[f] | 10.3 | * | * | * |
| *S. griseus* S104 | * | * | 9.3-10.3 | 8.3-10.3 | * |
| *S. vinaceus* S208 | * | * | * | * | 8.3 |
| *Nocardia* sp. 52 | – | 5.51-6.38 | – | 2.3-6.3 | 4.3 |
| *N. farcinica* 378[g] | – | – | – | – | – |

[a]Also MLSP1, MLSP2, $V_{104}$, and WSP4.
[b]Also MSP6.
[c]Also WSP5 and MSP5.
[d]Also MSP8 and MNP3.
[e]Also MSP22.
[f]Log number of plaque-forming units; (-) resistance of the actinomycete to actinophage, titer less than 20; (*) not tested.
[g]Also *N. rubra* strains 1 and 74, *N. opaca* 76, *J. canicruria* 57, *N. corallina* strains 78 and 107, *M. rhodochrous* strains 237 and 370, *Nocardia* sp. 54, *N. asteroides* strains 58, 73, 84, 92, 96, 133, 155, and 162, *N. farcinica* strains 378S, 1362, and 2089, *M. fortuitum* 235, *M. smegmatis* 238, *M. platypoecilus* 274, *M. smegmatis* A404, *M. rabinowitschi* A402, *M. fribergensis* A403, *Mycobacterium* sp. A405, and *M. stercoides* A406.

TABLE 2. *Susceptibilities of nocardiae, mycobacteria, and other actinomycetes to actinophages*

| Host | Phage | | | |
|---|---|---|---|---|
| | MVP1[a] | MVP2 | MMP1 | MSP10[b] |
| *S. lavendulae* 547 | *[c] | 8.7 | * | * |
| *S. cinnamomeus* 1285 | 8.3 | * | * | * |
| *S. violaceoruber* S199 | * | * | * | 7.7-8.7 |
| *M. rhodochrous* 370 | – | – | – | – |
| *Nocardia* sp. 52 | – | – | – | 4.3-8.18 |
| *N. farcinica* 378[d] | – | – | – | – |
| *Micromonospora* sp. A401 | * | * | 6.7-8.7 | * |

[a]Also MVP4 and MVP6.
[b]Also MSP7, MSP11, MSP19, and MVP3.
[c](*)Not tested; (–)resistance of the actinomycete to actinophage, titer less than 50; numbers are log numbers of plaque-forming units.
[d]See Table 1.

All five mycobacterium phages attacked strains of *Nocardia* (Table 3). *M. rhodochrous* 237 and *N. farcinica* strains 378, 378S, and N306 were each susceptible to two or more mycobacterium phages.

TABLE 3. *Susceptibilities of nocardiae, mycobacteria, and other actinomycetes to mycobacterium phages*

| Host | Phage | | | | |
|---|---|---|---|---|---|
| | MMP8 | MMP10 | MMP11 | MMP7 | MMP9 |
| *M. rabinowitschi* A402 | 8.7[a] | 7.7 | 6.7 | 7.42 | – |
| *M. fribergensis* A403 | 8.65 | 8.0 | 7.77 | 7.77 | 5.13 |
| *M. smegmatis* A404 | * | – | 6.05 | 7.81 | 3.31 |
| *Mycobacterium* sp. A405 | 8.85 | 9.06 | 8.07 | 8.17 | 7.77 |
| *M. stercoides* A406 | 2.7 | 8.7 | 7.95 | 7.33 | 4.4 |
| *N. farcinica* 378 | 2.78 | 8.4 | – | 2.77 | 2.25 |
| *N. farcinica* 378S | – | – | – | 3.07 | 2.85 |
| *N. farcinica* N306 | – | 2.7 | 3.77 | – | – |
| *M. rhodochrous* 237 | 5.18 | 8.7 | 2.7 | – | 5.77 |
| *M. rhodochrous* 370[b] | – | – | – | – | – |

[a]Log number plaque-forming units; (-) resistance of the actinomycete to actinophage, titer less than 50; (*) not tested.

[b]Also *N. rubra* strains 1 and 74, *N. opaca* 76, *J. canicruria* strains 57 and 1574, *N. corallina* strains 78, 107, N305, 2747, N307, 4273, and S5, *Nocardia* sp. 52, *Nocardia* sp. 54, *N. asteroides* strains 58, 73, 84, 92, 96, 133, 155, and 162, *N. farcinica* strains 1362 and 2089, *Nocardia* sp. 3403, *N. brasiliensis* N301, *M. fortuitum* 235, *M. smegmatis* 238, and *M. platypoecilus* 274.

TABLE 4. *Susceptibilities of nocardiae, mycobacteria, and other actinomycetes to actinophages isolated on N. brasiliensis, J. canicruria, and M. rhodochrous 370*

| Host | Phage | | | |
|---|---|---|---|---|
| | MNP1[a] | MJP1 | MNP8 | MMP4[b] |
| *N. brasiliensis* N301 | 8.3[c] | 6.7-8.7 | – | – |
| *J. canicruria* 1574R | * | 9.7 | * | * |
| *J. canicruria* 1574 | 6.3-7.3 | 4.7-5.7 | 2.22-3.22 | 6.0-9.0 |
| *J. canicruria* 57 | – | 8.7 | 3.22-4.22 | * |
| *N. corallina* W5 | – | * | 11.22 | – |
| *N. corallina* 78 | – | – | 8.22-11.22 | * |
| *N. corallina* N305 | * | 9.65 | – | * |
| *N. corallina* 2747[d] | * | – | 8.92-11.22 | * |
| *N. corallina* 4273[e] | * | * | 8.67-9.25 | * |
| *N. opaca* 76 | – | 4.48 | 8.0 | * |
| *M. rhodochrous* 370 | – | 6.7-8.7 | 3.22 | 9.0 |
| *N. asteroides* 96 | 5.1-6.0 | 6.7-9.7 | – | * |
| *N. farcinica* 378 | –[f,g] | –[f,g] | –[f,g] | –[g] |

[a]Also MNP2 and MNP7.

[b]Also MMP5 and MMP6.

[c]Log number of plaque-forming units; (–) resistance of the actinomycete to actinophage, titer less than 50; (*) not tested.

[d]Also *N. corallina* N307.

[e]Also *N. corallina* S5.

[f]Also *N. rubra* strains 1 and 74, *Nocardia* sp. 52, *Nocardia* sp. 54, *N. corallina* 107, *N. asteroides* strains 58, 73, 84, 92, 133, 155, and 162.

[g]Also *N. farcinica* strains 378S, 1362, and 2089, *M. fortuitum* 235, *M. rhodochrous* 237, *M. smegmatis* 238, *M. platypoecilus* 274, *M. rabinowitschi* A402, *M. fribergensis* A403, *M. smegmatis* A404, *Mycobacterium* sp. A405, and *M. stercoides* A406.

*M. rhodochrous* 370 and eight strains of *N. asteroides*, however, were among the organisms unaffected by all five mycobacterium phages. Conversely, actinophages MNP1, MNP2, and MNP7, all of which were isolated on *N. brasiliensis* N301, attacked *N. asteroides* 96 and *J. canicruria* 1574 but were unable to lyse true mycobacteria or organisms designated as *N. farcinica* (Table 4). Moreover, actinophage MNP6 attacked *N. asteroides* strains 92 and 96 as well as *N. brasiliensis* N301 and *J. canicruria* 1574.

Significantly, strains of *N. corallina, N. opaca, M. rhodochrous, J. canicruria, N. asteroides*, and *N. brasiliensis* served as alternate hosts for *Jensenia* phage MJP1. The susceptibilities of *M. rhodochrous* 370, *N. opaca* 76, six strains of *N. corallina*, and two strains of *J. canicruria* to actinophage MNP8 constituted additional evidence for the close relationship of these organisms. Three phages isolated on *M. rhodochrous* 370 were unable to destroy tested strains of *N. farcinica*, true mycobacteria, and *M. rhodochrous* 237.

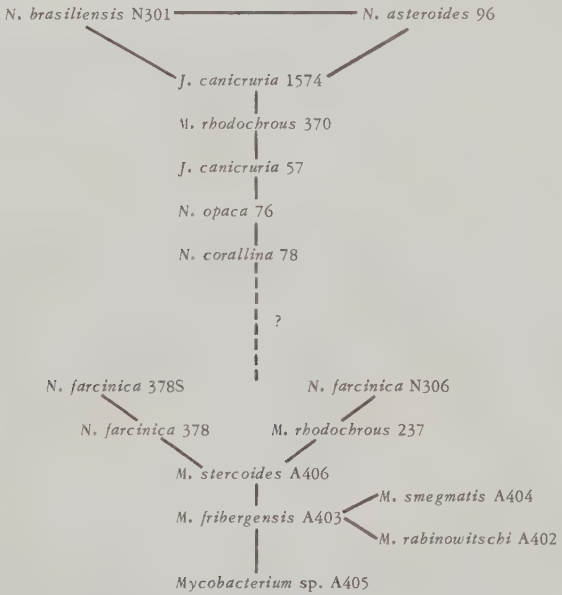


FIG. 1. *Relationship among actinomycete hosts on the basis of susceptibility to actinophages. Length of the branches is not significant.*

The strains used in the present study were grouped on the basis of common viral sensitivity and efficiency of plating of the phages upon alternative hosts (Fig. 1). For example, *N. asteroides* 96 and

*N. brasiliensis* N301 were distinguishable because the former microbe gave fewer plaque-forming units for a given challenge of actinophage MNP1, MNP2, or MNP7 than the latter organism (Table 4), *J. canicruria* 1574 was closely allied to both *N. asteroides* 96 and *N. brasiliensis* N301 in that it was lysed by all viruses attacking these two species and by one additional phage. Similarly, *M. rhodochrous* 370, *J. canicruria* 57, *N. opaca* 76, and *N. corallina* 78 were related to one another and to *J. canicruria* 1574 on the basis of sensitivity to actinophages and their efficiency of plating.

Actinomycetes resistant to all actinophages, including six strains of *N. asteroides*, two strains each of *N. farcinica* and *N. rubra*, three strains of *Mycobacterium* and *N. corallina* 107, could not be placed in the phylogenetic sequence.

## Discussion

Most of the actinomycetes used in this study have been shown to be related to one another to varying degrees. Sharp boundaries between genera of *Actinomycetales*, however, are nonexistent. Both qualitative and quantitative determination of viral susceptibility seems to be useful for establishing general phylogenetic relationships among actinomycetes, with an accuracy equivalent to other systems available. Viruses able to attack actinomycetes resistant to available phages are needed to determine the phylogenetic status of these organisms. Mislabeling of strains is one perplexing problem generally associated with phage typing and taxonomy. *Nocardia* sp. 52 studied here, and *N. paraguayensis* and *N. madurae* studied earlier, are properly streptomycetes, not nocardiae. Phage typing is subject to several other complications, including contamination of typing reagents with temperate phages and antibiotics, host modification of virulence, and numerous technical difficulties. Most of these problems can be circumvented by using dilution series of clean, high-titered lysates and by carefully comparing assay cultures with control plates. Density of the host inoculative suspension, physical conditions, and composition of the test medium are important considerations.

Interpretation of host-range data is difficult at times. Relationships among some actinomycetes are not apparent immediately from the data, hence strains are grouped on the basis of common sensitivity to individual viruses (overlapping viral susceptibilities) or on the basis of quantitative aspects of susceptibility (efficiency of plating). The total number of qualitatively identical reactions of hosts with respect to a series of phages is another means for detecting similarities.

The following phylogenetic scheme for soft nocardiae and related actinomycetes is suggested: *N. brasiliensis* N301—*J. canicruria* 1574—

*M. rhodochrous* 370—*J. canicruria* 57—*N. opaca* 76—*N. corallina* 78. Note that *M. rhodochrous* 370 has been placed between two strains of *J. canicruria* and that *N. opaca* 76 has been placed in this group. It seems apparent that *N. corallina*, *N. opaca*, *J. canicruria*, and *M. rhodochrous* 370 are rightfully members of the same genus.

*N. asteroides* 96 was found to be closely allied to *N. brasiliensis* N301; however, most of the *N. asteroides* strains studied were resistant to all tested actinophages and could not be placed either with the mycobacteria or the soft nocardiae.

Of special significance was the finding that three strains of *N. farcinica*, the type species of the genus *Nocardia*, were attacked by phages virulent for true mycobacteria which were acid-fast and nonchromogenic. Thus it appears that the biological affinities of certain strains designated *N. farcinica* are with the true mycobacteria. Moreover, *M. rhodochrous* 237 was placed in this group rather than with the soft nocardiae of which *M. rhodochrous* 370 was a member. *N. farcinica* and *N. asteroides*, often considered as synonymous, were assigned to separate groups on the basis of susceptibility to phage. Additional phages must be tested to determine the relationship, if any, between these two important species. It should be noted that although the majority of the phage tests were negative, negative findings were in some instances quite significant.

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## Literature Cited

Alexander, R. R., and E. McCoy. 1956. Characterization of *Streptomyces griseus* bacteriophages. J. Bacteriol. **72**:378-386.

Anderson, D. L. 1961. Studies on actinophage. Ph.D. Thesis, University of Minnesota, Minneapolis.

Bradley, S. G., and D. L. Anderson. 1958. Taxonomic implication of actinophage host range. Science **128**:413-414.

Bradley, S. G., D. L. Anderson, and L. A. Jones. 1961. Phylogeny of actinomycetes as revealed by actinophage susceptibility. Develop. Indust. Microbiol. **2**:223-237.

Felix, A. 1956. Phage typing of *Salmonella typhimurium*: its place in epidemiological and epizootiological investigations. J. Gen. Microbiol. **14**:208-222.

Gilmour, C. M., E. C. Noller, and B. Watkins. 1959. Studies on Streptomyces phage. I. Growth characteristics of the *Streptomyces griseus* host-phage system. J. Bacteriol. **78**:186-192.

Welsch, M., R. Corbaz, and L. Ettlinger. 1957. Phage typing of streptomycetes. Schweiz. Z. allgem. Pathol. u. Bakteriol. **20**:454-458.

# ANTIBIOTIC COMBINATIONS: AN IN VITRO STUDY OF ANTISTAPHYLOCOCCAL EFFECTS OF ERYTHROMYCIN PLUS PENICILLIN, STREPTOMYCIN, OR TETRACYCLINE

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For several years this laboratory has been interested in the in vitro inhibitory effect of antibiotic combinations on microorganisms implicated in infectious processes in man. Earlier work (Welch et al., 1958; Reedy et al., 1958; Oswald and Welch, 1958) yielded interesting information but no outstanding examples of active inhibitory combinations.

Recently, Herrell, Balows, and Becker (1960) and Herrell (1961) reported that a combination of propionyl erythromycin and penicillin consistently exerted significant inhibitory action in vitro against staphylococci that were resistant to the antibiotics when tested separately. The present study was undertaken in an attempt to corroborate these findings and see if the same effect could be obtained with erythromycin and other antibiotics.

## Materials and Methods

Herrell et al. (1960, 1961) determined the activity of propionyl erythromycin-penicillin mixtures composed of a fixed 1:1 proportion of each ($\mu$g:units). Since it is likely that the optimum combined effect might occur with ratios other than this, a test design was sought which would furnish a number of different proportions. Therefore, a two-dimensional, twofold serial dilution pattern was used, making it possible to determine more precisely the minimal concentrations of mixed antibiotics having inhibitory action against a particular culture.

Although Herrell et al. (1960, 1961) used propionyl erythromycin as one component of the mixtures tested, erythromycin estolate (propionyl erythromycin lauryl sulfate) and erythromycin base were used

in the present study. Erythromycin base is the fundamental moiety from which the various esters derive their antibiotic activity, even though the derivatives may exert an antibacterial effect as complete entities without hydrolyzing to free erythromycin base. Erythromycin estolate was chosen because it has largely replaced propionyl erythromycin as the article of commerce.

In the reports of Herrell et al. (1960, 1961), the particular type of penicillin used in the in vitro studies was not specified, although penicillin V was used in the clinical studies. Potassium penicillin G was used throughout the present work.

A total of 16 coagulase-positive clinical isolates of *Staphylococcus aureus* was used in this study. Of the isolates, 12 were resistant in vitro to erythromycin, penicillin, streptomycin, and tetracycline, and 4 were susceptible to these antibiotics.

The 12 resistant strains were tested for susceptibility to erythromycin estolate (EE), erythromycin base (EB), potassium penicillin G (KG), streptomycin (SM), and tetracycline (TC) alone, and to the following mixtures: EE + KG, EB + KG, EE + SM, EB + SM, EE + TC, and EB + TC.

The four susceptible strains were tested against erythromycin and penicillin G, separately and in combination.

*Determination of antimicrobial activity of antibiotic combinations.* Solutions of each antibiotic were prepared in Trypticase soy broth to give concentrations ranging in twofold increments from 2 to 512 $\mu$g per ml for testing the resistant organisms, and from 0.002 to 0.5 $\mu$g per ml for the susceptible ones.

To study the antimicrobial activity of a given pair of antibiotics (A and B, for example), 100 sterile, 100 × 13 mm test tubes were arranged in a 10 × 10 design. To each tube in the first horizontal row and in the first vertical column was added 0.5 ml of sterile, antibiotic-free Trypticase soy broth. To each tube in the second horizontal row was added 0.5 ml of the lowest concentration of antibiotic A; then each horizontal row from row 3 to row 10 was similarly treated, but with each successive row the next higher concentration of antibiotic A in the series was used. To each tube in the second vertical column was added 0.5 ml of the lowest concentration of antibiotic B. Each vertical column from column 3 to column 10 was similarly treated, but with each successive column the next higher concentration of antibiotic B in the series was used. Each tube, thus containing 1.0 ml of medium, was inoculated with 1.0 ml of a 1:1,000 dilution of a 24-hr culture of the organism being treated.

The resulting arrangement consisted of one tube with no antibiotic, and serial twofold dilutions of antibiotic A alone in the first vertical column, and antibiotic B alone in the first horizontal row. The remaining tubes contained amounts of both antibiotics in varying proportions.

The tubes were incubated in a water bath at 37 C for 16 to 18 hr. The tubes were inspected and those noted in which there was complete inhibition of the organism.

## Results

To illustrate the inhibitory effect of combined antibiotics as compared to the individual components, results of two typical experiments are shown in Tables 1 and 2.

TABLE 1. *Typical experiment showing inhibitory effects of combinations of erythromycin and penicillin G on a resistant Staphylococcus aureus, JHP 29R**

| Penicillin G (μg/ml) | Erythromycin (μg/ml) | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | 0 | 0.5 | 1 | 2 | 4 | 8 | 16 | 32 | 64 | 128 |
| 0 | + | + | + | + | + | + | + | + | + | + |
| 0.5 | + | + | + | (−) | − | − | − | − | − | − |
| 1 | + | + | + | − | − | − | − | − | − | − |
| 2 | + | + | + | − | − | − | − | − | − | − |
| 4 | + | + | (−) | − | − | − | − | − | − | − |
| 8 | + | + | − | − | − | − | − | − | − | − |
| 16 | + | + | − | − | − | − | − | − | − | − |
| 32 | + | + | − | − | − | − | − | − | − | − |
| 64 | + | − | − | − | − | − | − | − | − | − |
| 128 | + | − | − | − | − | − | − | − | − | − |

*The top horizontal row represents susceptibility to erthromycin alone (256 μg or more per ml); left vertical column represents susceptibility to penicillin G alone (256 μg or more per ml). MIC's of mixtures (μg/ml): erythromycin plus penicillin G − (1 + 4) or (2 + 0.5). Symbols: + = growth in tube; − = no visible growth.

TABLE 2. *Typical experiment showing inhibitory effects of combinations of erythromycin and penicillin G on a sensitive Staphylococcus aureus, JHP 12R**

| Penicillin G (μg/ml) | Erythromycin (μg/ml) | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | 0 | 0.0005 | 0.001 | 0.002 | 0.0039 | 0.0078 | 0.0156 | 0.0312 | 0.062 | 0.125 |
| 0 | + | + | + | + | + | + | + | + | + | + |
| 0.0005 | + | + | + | + | + | + | + | + | + | + |
| 0.001 | + | + | + | + | + | + | + | + | + | + |
| 0.002 | + | + | + | + | + | + | + | + | + | + |
| 0.0039 | + | + | + | + | + | + | + | + | + | + |
| 0.0078 | + | + | + | + | + | + | + | + | + | + |
| 0.0156 | + | + | + | + | + | + | + | (−) | − | − |
| 0.0312 | + | + | + | + | + | + | + | − | − | − |
| 0.0625 | − | − | − | − | − | − | − | − | − | − |
| 0.125 | − | − | − | − | − | − | − | − | − | − |

*The top horizontal row represents susceptibility to erythromycin alone (equal to or greater than 0.25 μg/ml, but an actual value of 0.25 μg/ml was found in a previous test); left vertical column represents susceptibility to penicillin G alone (1.0625 μg/ml). MIC of inhibitory mixture: 0.0312 μg/ml of erythromycin plus 0.0156 μg/ml of penicillin. Symbols: see Table 1.

The test organism represented in Table 1 was resistant to 128 $\mu$g per ml of both erythromycin and penicillin. Thus, if the series of twofold concentrations tested had been continued, the minimal inhibitory concentration (MIC) of each would have been observed as at least 256 $\mu$g per ml or possibly as 512 $\mu$g per ml or greater. Among the mixtures tested, two were notably active. One consisted of 1 $\mu$g of erythromycin and 4 $\mu$g of penicillin G per ml and the other of 2.0 and 0.5 $\mu$g, respectively. By expressing these values as fractional parts of an MIC for each component antibiotic (Welch et al., 1958), it is apparent that the first inhibitory mixture contained 1/256 of an MIC of erythromycin and 1/64 of an MIC of penicillin. The other inhibitory mixture contained 1/128 of an MIC of erythromycin and 1/512 of an MIC of penicillin. Since the first mixture required a sum of 5/256 fractional parts of an MIC and the second only 5/512, it would appear that the latter is more efficient.

There were, of course, numerous other less efficient combinations which were, nevertheless, quite active. For example, the following $\mu$g/ml concentrations of erythromycin plus penicillin were inhibitory: 2:1, 2:2, 2:4, 4:0.5, 4:1, 4:2, and 4:4.

The results of a typical experiment, in which the strain of *S. aureus* was quite susceptible to both erythromycin and penicillin G, are shown in Table 2. These results are markedly less striking than those obtained with the doubly resistant strain in Table 1, since 1/8 of an MIC of erythromycin combined with 1/4 of an MIC of penicillin achieved the same inhibitory result obtained with either antibiotic alone.

*Activity of mixtures.* The results of 75 experiments with five antibiotic products are given in Table 3. When evaluated according to the criteria to be described, some combinations of antibiotics were considerably more efficient than was expected from the activity of the individual components. Each MIC has been expressed as the $\log_2$ of the amount, as recommended by the World Health Organization Expert Committee on Antibiotics (1961). For example, for the first organism, JHP2R, the MIC's of the individual antibiotics were 64 $\mu$g per ml ($2^6$) of erythromycin estolate and 256 $\mu$g per ml ($2^8$) of penicillin G. In combination, only 8 $\mu$g per ml ($2^3$) of each was required for inhibition. The fractional parts of an MIC may be quickly determined by inspection of the table. If the $\log_2$ value of the MIC of the antibiotic in combination is 1 less than for the antibiotic alone, the fractional part is 1/2. Similarly, for differences of 2, 3, 4, 5, etc., the fractional parts are 1/4, 1/8, 1/16, 1/32, etc.

The sum of the fractional parts of the MIC's for the components of a mixture may be expressed as the percentage of the expected sum (1). For example, culture JHP2R was inhibited by 1/8 of an MIC of erythromycin estolate combined with 1/32 of an MIC of penicillin. The sum of these fractional parts, 5/32, is 15.6% of 1.

TABLE 3. *MIC's* of erythromycin and penicillin, streptomycin, or tetracycline singly and in combination against 16 Staphylococcus aureus cultures*

| Resistant strains | EE | KG | EE+KG | EB | KG | EB+KG | EE | SM | EE+SM | EB | SM | EB+SM | EE | TC | EE+TC | EB | TC | EB+TC |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| JHP 2 R | 6 | 8† | 3 3 | 8 | 8 | 1 −1 | 7 | 8 | 2 5 | 8 | 8 | 0 5 | 6 | 7 | 2 2 | 8 | 7 | 2 2 |
| 5 R | 6 | 8 | 2 1 | 8 | 8 | 1 −1 | 6 | 8 | 4 4 | 8 | 8 | 3 4 | 6 | 7 | 2 2 | 8 | 6 | 1 3 |
| 17 L | 5 | 8 | 2 2 | 8 | 8 | 2 −1 | 5 | 8 | 4 3 | 8 | 8 | 1 5 | 5 | 7 | 2 4 | 8 | 7 | 2 3 |
| 29 R | 6 | 8 | 2 2 | 8 | 8 | 1 −1 | 6 | 8 | 4 4 | 8 | 8 | 1 5 | 6 | 7 | 2 4 | 8 | 7 | 2 3 |
| 30 R | 6 | 8 | 3 4 | 8 | 6 | 1 −1 | 7 | 8 | 4 4 | 8 | 8 | 1 5 | 6 | 6 | 2 3 | 8 | 6 | 2 3 |
| 64 R | 6 | 8 | 3 2 | 8 | 8 | 1 2 | 6 | 8 | 3 4 | 8 | 8 | 1 4 | 6 | 7 | 1 4 | 8 | 7 | 3 3 |
| 75 R | 5 | 8 | 4 3 | 8 | 8 | 2 1 | - | - | - - | 8 | 8 | 1 5 | 6 | 7 | 3 3 | 8 | 7 | 3 3 |
| 78 R | 6 | 8 | 4 2 | 8 | 8 | 1 1 | 6 | 8 | 3 4 | 8 | 8 | 1 5 | 6 | 7 | 3 3 | 8 | 7 | 3 3 |
| JHS 3 L | 6 | 8 | 3 3 | 8 | 8 | 1 1 | 6 | 8 | 4 4 | 8 | 8 | 2 5 | 6 | 7 | 2 3 | 8 | 7 | 3 3 |
| 14 R | 6 | 8 | 3 4 | 8 | 8 | 1 1 | 6 | 8 | 4 4 | 8 | 8 | 2 5 | 6 | 7 | 3 3 | 8 | 7 | 3 3 |
| 17 R | 5 | 8 | 3 2 | 8 | 8 | 1 1 | 8 | 6 | 6 5 | 8 | 8 | 1 5 | 6 | 6 | 2 4 | 8 | 8 | 1 3 |
| 31 L | 6 | 8 | 2 1 | 8 | 8 | 1 −1 | 6 | 8 | 2 4 | 8 | 8 | 2 5 | 6 | 6 | 2 3 | 8 | 8 | 3 3 |
| **Sensitive strains** | | | | | | | | | | | | | | | | | | |
| JHP 11 R | | | | −3 | −4 | −4 −6 | | | | | | | | | | | | |
| 12 R | | | | −2 | −4 | −5 −6 | | | | | | | | | | | | |
| 17 R | | | | −4 | −2‡ | −7 −6 | | | | | | | | | | | | |
| 38 R | | | | −3 | −4 | −3 −6 | | | | | | | | | | | | |

*Minimal inhibitory concentration expressed as $\log_2$ of the $\mu$g/ml; thus, 0 denotes 1 $\mu$g/ml and 1, 2, 3, 4, etc. denote 2, 4, 8, 16, etc., $\mu$g/ml and −1, −2, −3, −4, etc. denote 0.5, 0.25, 0.125, 0.0625, etc.

†All values of 8 should be expressed as "equal to or greater than."

‡A value of −4 was obtained in a previous test.

TABLE 4. *Antibiotic combinations tested against antibiotic-resistant staphylococci*

| Combination | Inhibitory amounts* | Cultures inhibited |
|---|---|---|
| | % | *number* |
| EE + KG | 7.0 | 2 |
| | 14.0 to 18.7 | 7 |
| | 26.6 | 2 |
| | 53.1 | 1 |
| EE + SM | 12.5 to 18.7 | 5 |
| | 28.1 to 31.2 | 5 |
| | 75.0 | 1 |
| EE + TC | 9.4 | 2 |
| | 12.5 to 18.7 | 8 |
| | 25.0 to 31.2 | 2 |
| EB + KG | 1.0 | 4 |
| | 1.6 | 5 |
| | 1.8 to 2.3 | 3 |
| EB + SM | 9.4 | 1 |
| | 12.5 to 14.0 | 11 |
| EB + TC | 3.9 to 4.7 | 2 |
| | 6.3 to 9.4 | 8 |
| | 13.3 to 14.0 | 2 |

*Expressed as the percentage of the expected amount.

The results in Table 3 show that when various mixtures of antibiotics were tested against 12 antibiotic-resistant staphylococci, in many instances the combined amount of antibiotics required for inhibitory action was considerably less than would be expected. The percentages of the expected amounts that were inhibitory are given in Table 4.

When erythromycin and penicillin G were tested against four susceptible staphylococci, the following percentages of the expected amount were inhibitory: 18.7, 37.5, 75, and 125.

## Discussion

It is obvious that, with the combinations of antibiotics tested, the amount of each antibiotic required to inhibit the resistant organisms was strikingly smaller than the MIC of the individual components tested alone.

The mixtures of erythromycin base with penicillin G, streptomycin, or tetracycline seem to be inhibitory at lower concentrations and with smaller total fractional parts of an MIC than were mixtures of erythromycin estolate with these antibiotics.

The mixtures of erythromycin base with penicillin G were particularly outstanding for inhibitory action at low concentrations. The fact that smaller total fractional parts of an MIC were required by mixtures containing erythromycin base as compared to those containing erythromycin estolate may be explained partially by the fact that

the cultures were more resistant to erythromycin base. However, in almost every case, inhibition was achieved with less erythromycin base plus penicillin than erythromycin estolate plus penicillin.

These findings confirm those of Herrell et al. (1960, 1961) with regard to the inhibitory action of erythromycin-penicillin mixtures against staphylococci resistant to both when tested separately. The same phenomenon was observed to a lesser degree with erythromycin plus streptomycin and erythromycin plus tetracycline.

There appears to be some conflict between these findings and the view that antibiotics in pairs have little or no combined effect on bacterial growth, if the strain of staphylococcus is resistant to either antibiotic (Elliott and Hall, 1959). However, the present observations are most unusual in that the organisms tested were resistant to both antibiotics. In a report from this laboratory on oleandomycin mixtures the results seemed to indicate that synergism was encountered three times more frequently when the organism was sensitive to both antibiotics than when it was resistant to one or both (Welch et al., 1958).

Manten (1954) concluded that erythromycin-penicillin mixtures were frequently antagonistic, based on a replica plating method in which the bactericidal action of penicillin alone became bacteriostatic only in the presence of erythromycin. The present study was not hampered by the difficulties inherent in diffusion methods, and as previously emphasized, the organisms were resistant to both antibiotics in each mixture.

## Literature Cited

Elliott, H. J., and W. H. Hall. 1959. A comparison of erythromycin with oleandomycin in combination with other antibiotics against hospital staphylococci. J. Lab. Clin. Med. 53:364-375.

Herrell, W. E. 1961. Erythrocillin in the treatment of infections due to Staphylococcus aureus. Virginia Med. Monthly 88:125-131.

Herrell, W. E., A. Balows, and J. Becker. 1960. Erythrocillin: A new approach to the problem of antibiotic-resistant staphylococci. Antibiotic Med. & Clin. Therapy 6:637-642.

Manten, A. 1954. Synergism and antagonism between antibiotic mixtures containing erythromycin. Antibiotics & Chemotherapy 4:1128-1233.

Oswald, E. J., and H. Welch. 1958 Antibiotic combinations. III. In vitro effects of oleandomycin-tetracycline on two hundred and two cultures of *Micrococcus pyogenes* var *aureus*. Antibiotics Ann. 1957-58, p.751-755.

Reedy, R. J., W. W. Wright, E. J. Oswald, and M. Ostrolenk. 1958. Antibiotic combinations: In vitro effects on selected groups of bacteria. II. Combinations of chloramphenicol with sixteen other antibiotics. Antibiotics Ann. 1957-58, p.745-750.

Welch, H., W. W. Wright, R. J. Reedy, and D. M. Wintermere. 1958. Antibiotic combinations: In vitro effects on selected groups of bacteria. I. Combinations of oleandomycin with sixteen other antibiotics. Antibiotics Ann. 1957-58, p.738-744.

World Health Organization. Expert Committee on Antibiotics. 1961. 2nd report. Standardization of methods for conducting microbic sensitivity tests. Technical report series. No. 210.

# SELECTIVE MEDIA FOR STAPHYLOCOCCI: COMPARISON OF POLYMYXIN, SORBIC ACID, AND COMBINATIONS THEREOF

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Infection and cross-infection with staphylococci are very serious problems today. Management of these infections and epidemiological investigations would be facilitated by a reliable selective and differential medium. Previously available media have had a number of drawbacks, including inconsistent results, poor selectivity, atypical morphology, and partial inhibition or delayed growth of *Staphylococcus aureus*. A polymyxin medium offering advantages over other selective media has previously been reported from this laboratory (Finegold and Sweeney, 1961*a*, *b*). Stuart (1959) had noted that surface impregnation of media with polymyxin facilitated recovery of *S. aureus* (as well as of streptococci and pneumococci) from throat swabs. The incorporation of 1% polyoxyethylene sorbitan monooleate (Tween 80) in the polymyxin medium (Finegold and Sweeney, 1961*b*) helps growth and pigmentation of *S. aureus* somewhat and may be useful in other ways, since Dye (1959) noted that 1% Tween 80 (added to Trypticase soy agar) allowed G-colony variants of staphylococci to grow as large colonies, and Erlandson and Lawrence (1953) noted that Tween 80 inactivated hexachlorophene-type compounds, some substituted phenolic disinfectants, and certain quaternary ammonium surface-active germicides. The polymyxin medium has been further modified (Finegold and Sweeney, *unpublished data)* by the addition of soy lecithin (The Glidden Company, Chicago, Ill.) in a concentration of 0.7 g/liter to achieve better inactivation of quaternary ammonium germicides (Quisno, Gibby, and Foter, 1946).

Raj and Liston (1961) have described a promising sorbic acid medium; the usefulness of this medium would be limited to certain applications since it is liquid.

The present study compares a solid version of the sorbic acid medium, the polymyxin medium (75 $\mu$g/ml), and various other media

incorporating varying concentrations of sorbic acid, varying concentrations of polymyxin, and combinations thereof.

## Materials and Methods

*First laboratory trial.* Comparisons were made between a sorbic acid solid medium, the standard polymyxin medium (75 μg/ml), sorbic acid medium plus 75 μg/ml of polymyxin, the polymyxin medium plus 0.15% sorbic acid with a final pH of 6.7 to 7.0, and nutrient agar. The percentage composition of the sorbic acid medium is as follows: sorbic acid, 0.15; thioglycolic acid, 0.03; cystine, 0.025; bromothymol blue, 0.001; yeast extract, 0.5; tryptose, 1.5; sodium chloride, 4.5; mannitol, 1.0; agar, 1.5; and distilled water to volume (final pH 6.7 to 7.0). The composition of the standard polymyxin medium is as follows: nutrient agar; polymyxin (75 μg or 750 units base activity per ml); cycloheximide 400 μg/ml (this ingredient is optional); Tween 80, 1%; and soy lecithin, 0.7 g/liter. The media were autoclaved and poured into sterile petri dishes. Polymyxin was kindly supplied by Chas. Pfizer & Co., New York, N.Y., and cycloheximide by The Upjohn Company, Kalamazoo, Mich. It seems to be desirable to use deionized distilled water in making up the polymyxin medium.

Organisms used were stock laboratory strains which had been isolated within the past year from clinical infections, from carriers, or from the hospital environment. A total of 22 strains of *S. aureus* and 14 of coagulase-negative forms (*S. epidermidis* or micrococci) were utilized. The coagulase-negative strains were ones which had survived on the polymyxin medium in routine use. These strains were grown in brain heart infusion broth for 24 hr, diluted to $10^{-5}$, and then streaked on plates of the above-mentioned test media.

Plates were incubated for 24 hr at 37 C and then colony counts were made.

*Second laboratory trial.* The following 25 media were compared in this trial:

1. Nutrient agar + soy lecithin (0.7 g/liter), 1% Tween 80, and cycloheximide (0.4 g/liter).
2. Medium 1 + 0.2% sorbic acid.
3. Medium 1 + 0.15% sorbic acid.
4. Medium 1 + 0.1% sorbic acid.
5. Medium 1 + 0.05% sorbic acid.
6. Sorbic acid medium (as detailed in first laboratory trial).
7. Medium 6 + lecithin, Tween 80, and cycloheximide as in medium 1.
8. Medium 7 + 75 μg/ml of polymyxin.
9. Medium 7 + 50 μg/ml of polymyxin.
10. Medium 7 + 25 μg/ml of polymyxin.
11. Standard polymyxin medium (75 μg/ml).
12. Medium 11 + 0.2% sorbic acid.

13. Medium 11 + 0.15% sorbic acid.
14. Medium 11 + 0.1% sorbic acid.
15. Medium 11 + 0.05% sorbic acid.
16. Polymyxin medium with concentration of polymyxin reduced to 50 μg/ml.
17. Medium 16 + 0.2% sorbic acid.
18. Medium 16 + 0.15% sorbic acid.
19. Medium 16 + 0.1% sorbic acid.
20. Medium 16 + 0.05% sorbic acid.
21. Polymyxin medium with concentration of polymyxin reduced to 25 μg/ml.
22. Medium 21 + 0.2% sorbic acid.
23. Medium 21 + 0.15% sorbic acid.
24. Medium 21 + 0.1% sorbic acid.
25. Medium 21 + 0.05% sorbic acid.

Cycloheximide was incorporated in the nutrient agar and polymyxin media in a concentration of 400 μg/ml. The final pH of all media was 6.7 to 7.0.

The sources of test organisms are as noted in the first laboratory trial. Included were strains of the following: *S. aureus*, 26; coagulase-negative forms (*S. epidermidis* or micrococci), 25; *Bacillus* sp., 15; enterococci, 5; Viridans group or nonhemolytic streptococci, 6; *Proteus mirabilis*, 2; *P. morganii*, 4; *P. rettgeri*, 3; *P. inconstans*, 1; *Klebsiella-Aerobacter*, 2; *Escherichia coli*, 2; and 4 strains of *Pseudomonas*. In this trial we did not purposely select coagulase-negative strains which were resistant to polymyxin (as was done in the first laboratory trial). Organisms were grown in brain heart infusion broth for 24 hr, and $10^{-4}$ and $10^{-6}$ dilutions were prepared.

Plates of test media were inoculated with the above dilutions of the test strains by means of a multiple-inoculator replicating apparatus (Steers, Foltz, and Graves, 1959) which allowed the plating of 32 strains per plate. The plates were then incubated for 24 hr at 37 C. After this, observations were made of colony counts, colony size, and colony color.

*Field trials.* Preliminary field trials were made employing 3 or 4 of the most promising media (judging from the studies with laboratory strains) in cultures of the hospital environment. Included were cultures of floors, of linen, and miscellaneous surfaces and objects. All of these cultures were obtained with cotton swabs. The swabs were then shaken in tubes of 5 ml of sterile normal saline (5 min on a Kahn shaker). After this the swabs were removed from the tubes, the contents of the tube were thoroughly mixed, and equivalent amounts (0.5 ml in one trial and 0.1 ml in another) were removed and put on the surface of the various test media. The inoculum was spread evenly by tilting the plates, and the plates were then incubated for 24 or 48 hr at 37 C. After incubation the different colony types were counted, subcultured, and identified.

## Results

Table 1 gives the results obtained in the first laboratory trial. Note particularly that the selected polymyxin-resistant coagulase-negative strains were inhibited considerably with the addition of sorbic acid when the standard polymyxin base was used.

TABLE 1. *Survival of staphylococci and micrococci on various media (first laboratory trial)*

| | Sorbic acid medium | Sorbic acid medium + polymyxin (75 μg/ml) | Polymyxin medium (75 μg/ml) | Polymyxin medium + 0.15% sorbic acid | Nutrient agar |
|---|---|---|---|---|---|
| Average count with 22 strains of *S. aureus* | 68.5 | 61.4 | 58.5 | 50.3 | 58.4 |
| Median count with 22 strains of *S. aureus* | 62.5 | 51.5 | 57.5 | 43.0 | 48.5 |
| Number of coagulase-negative strains showing growth (14 tested) | 14 | 14 | 13 | 6 | 14 |
| Average count of coagulase-negative strains (only those showing growth) | 61.2 | 59.8 | 40.5 | 16.3 | 49.5 |
| Median count of coagulase-negative strains (only those showing growth) | 50.0 | 55.0 | 23.0 | 11.0 | 41.5 |

Table 2 gives the results obtained with the strains of *S. aureus* on 12 of the media used in the second laboratory trial. Colony counts of *S. aureus* were generally comparable on all 25 media except that

TABLE 2. *Survival of Staphylococcus aureus on various media (26 strains, second laboratory trial)*

| Medium number* | No. strains failing to grow | Average† colony count | Median† colony count |
|---|---|---|---|
| 1 | 0 | 16.7 | 11.0 |
| 6 | 0 | 18.9 | 17.0 |
| 8 | 0 | 15.1 | 13.5 |
| 11 | 0 | 16.4 | 15.5 |
| 12 | 2 | 16.9 | 13.5 |
| 14 | 0 | 10.9 | 8.0 |
| 16 | 0 | 15.9 | 14.5 |
| 17 | 0 | 15.3 | 13.0 |
| 19 | 0 | 16.0 | 13.0 |
| 21 | 0 | 19.0 | 18.0 |
| 22 | 0 | 17.9 | 16.5 |
| 24 | 0 | 17.4 | 13.5 |

*For complete description of media, see text.
†Calculated only from strains showing growth.

two strains were completely inhibited by 75 μg/ml of polymyxin + 0.2% sorbic acid. The sorbic acid medium interfered with pigmentation (compared to nutrient agar), and polymyxin decreased pigmentation slightly. The sorbic acid medium decreased colony size markedly. Addition of 0.15 or 0.2% sorbic acid to polymyxin (50 or 75 μg/ml) media decreased colony size of *S. aureus*.

Table 3 gives data comparable to that of Table 2 for coagulase-negative strains. Polymyxin added to sorbic acid medium loses much of its efficiency although surviving strains were much smaller than usual. In a nutrient agar base, polymyxin in a concentration of 50 to 75 μg/ml inhibits completely most unselected coagulase-negative strains. At these concentrations of polymyxin, sorbic acid showed no additive effect against these particular strains. However, 25 μg/ml of polymyxin was quite ineffective and was enhanced by the addition of sorbic acid.

TABLE 3. *Survival of Staphylococcus epidermidis and micrococci on various media (25 strains, second laboratory trial)*

| Medium number* | No. strains failing to grow | Average† colony count | Median† colony count |
|---|---|---|---|
| 1 | 0 | 27.7 | 23.0 |
| 6 | 1 | 28.9 | 27.0 |
| 8 | 6 | 34.4 | 25.0 |
| 11 | 22 | 12.0 | 14.0 |
| 12 | 23 | 13.5 | 13.5 |
| 14 | 23 | 6.0 | 6.0 |
| 16 | 22 | 12.7 | 16.0 |
| 17 | 23 | 20.5 | 20.5 |
| 19 | 23 | 25.5 | 25.5 |
| 21 | 2 | 26.2 | 27.0 |
| 22 | 17 | 12.0 | 11.0 |
| 24 | 15 | 17.0 | 19.0 |

*For complete description of media see text.
†Calculated only from strains showing growth.

Sorbic acid is very active against *Bacillus* species regardless of the base and other ingredients used, although it is most active in the sorbic acid medium base. With the polymyxin medium (concentrations of either 50 or 75 μg/ml), addition of 0.1% or more sorbic acid results in total elimination of about one-third of the *Bacillus* strains and a distinct reduction in size of most of the rest.

Enterococci and other streptococci are distinctly inhibited or eliminated only on the sorbic acid medium (with or without added polymyxin, lecithin, and Tween 80); they persist on all other media. The same is true for all *Proteus* species; *P. mirabilis* does not spread on nutrient agar or any of the other media.

The two strains of *E. coli* grew only on nutrient agar with or without sorbic acid, except for one strain which grew slightly on sorbic acid medium with polymyxin (75 μg/ml). Nutrient agar (with or without sorbic acid) was inactive against *Klebsiella-Aerobacter;* other media were equally active in preventing or suppressing growth (one strain grew with a decreased colony count). The four strains of *Pseudomonas* were all completely inhibited on all media containing polymyxin; only one was eliminated by plain sorbic acid medium (Fig. 1).

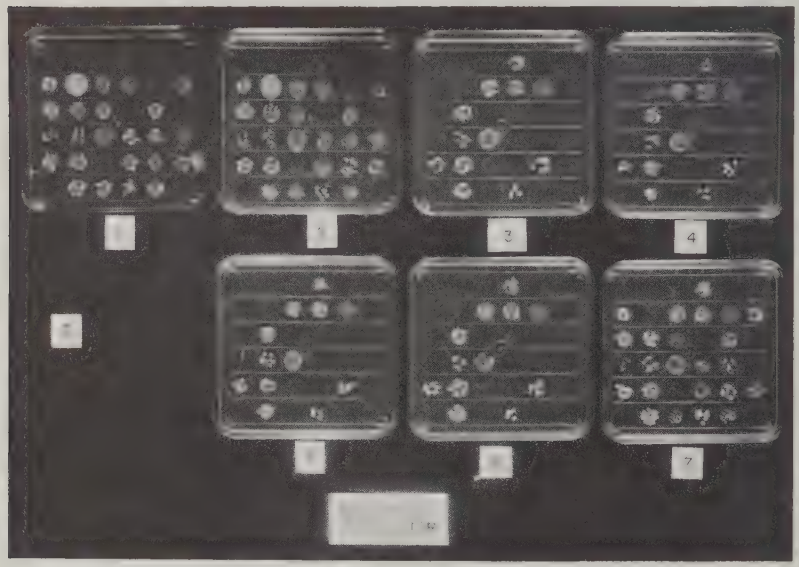

FIG. 1. *Survival of microorganisms on various media during second laboratory trial. Media used (see text): plate 1, no. 1; plate 2, no. 4; plate 3, no. 11; plate 4, no. 14; plate 5, no. 16; plate 6, no. 19; and plate 7, no. 21.*
*Top row left to right: S. viridans, S. viridans, S. aureus, Pseudomonas. Second row left to right: coagulase-negative, Klebsiella-Aerobacter, S. aureus, S. aureus, P. morgani, coagulase-negative. Third row left to right: coagulase-negative, S. aureus, coagulase-negative, Enterococcus, coagulase-negative. Fourth row left to right: coagulase-negative, S. aureus, P. rettgeri, coagulase-negative, coagulase-negative, E. coli. Fifth row left to right: coagulase-negative, S. aureus, enterococcus, coagulase-negative, S. aureus, coagulase-negative. Bottom row left to right: S. aureus, coagulase-negative, S. aureus, coagulase-negative.*

The field trials (carried out with polymyxin medium, 75 and 50 μg/ml, with and without 0.1% sorbic acid) were not satisfactory and will not be covered in detail. The plates inoculated with 0.5 ml remained wet, allowing some colonies to swarm together, and the plates inoculated with 0.1 ml contained too few organisms for reliable

comparisons. Nevertheless, we felt that these tests should be mentioned since they indicated the possibility that *S. aureus* in the environment might be inhibited by 0.1% sorbic acid added to polymyxin (in contrast to laboratory strains) and that therefore it may not be desirable to add the sorbic acid for certain applications of the polymyxin medium. With regard to elimination of unwanted organisms, the addition of sorbic acid to polymyxin media appeared to give good results; this was particularly striking in the case of *Bacillus* species (Fig. 2). The number of unwanted organisms increases with incubation beyond 24 hr.

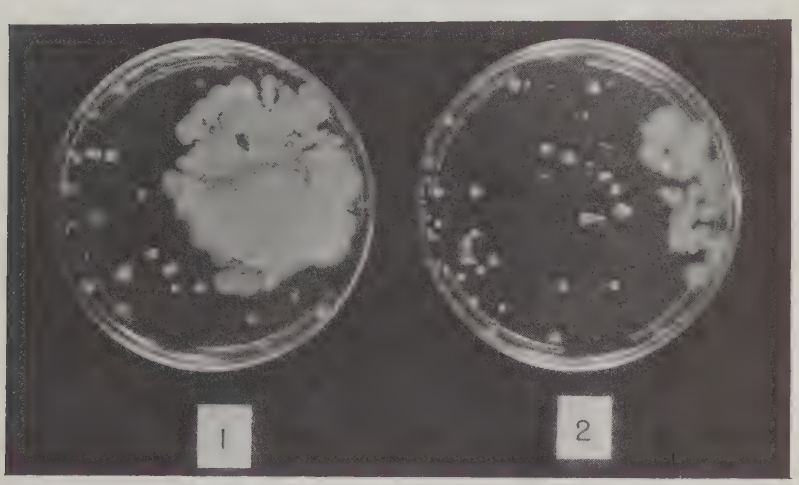


FIG. 2. *Results of floor cultures obtained during field trial using selected media. Plate 1 contains polymyxin medium (75 μg/ml); plate 2 contains polymyxin medium + sorbic acid (0.1%). Note marked suppression of Bacillus species with addition of sorbic acid; most of the other colonies are Staphylococcus aureus.*

## Discussion

The polymyxin medium (75 μg/ml, with soy lecithin, Tween 80, and cycloheximide) has been used for processing clinical specimens and for sampling the hospital environment for many months and has performed very well (Finegold and Sweeney, *unpublished data).* Although the majority of colonies recovered on this medium are ordinarily *S. aureus,* a number of other organisms are capable of growing on it. Certain characteristics will allow one to determine from gross inspection that some of these are definitely not *S. aureus* (Table 4).

Nevertheless, the medium could be improved by the addition of anything which would eliminate some or all of the persisting *S. epidermidis*, micrococci, diphtheroids, and *Bacillus* strains in particular.

TABLE 4. *Characteristics of various organisms surviving on polymyxin medium**

| Organism | Colony appearance-transmitted light | Color | Degree of growth | Average colony diameter |
|---|---|---|---|---|
| | | | | *mm* |
| *S. aureus* | Opaque | Usually cream to yellow | Good | 1.0 |
| *S. epidermidis* and micrococci | Opaque | Usually white | Considerable suppression | From pinpoint size to 0.6 |
| *Bacillus* species† | Opaque | White-cream | Some suppression | 3.0 - 8.0 or more |
| *Proteus mirabilis*‡ | Translucent | Blue-green (transmitted light) | Little suppression | 0.1 - 0.8 |
| Other *Proteus* species | Translucent | Blue-green (transmitted light) | Little suppression | 0.1 - 0.8 |
| Streptococci (Viridans, enterococci) | Usually translucent | Clear or white | Good | 0.1 - 0.6 |
| Diphtheroids | Often translucent Sometimes opaque | White-cream | Suppression | 0.1 - 0.6 |

*Polymyxin, 75 μg/ml; 24-hr reading.
†Characteristic rough appearance.
‡No spreading.

The possibility of improving the polymyxin medium by adding sorbic acid seemed reasonable. Blang and Ahsan (1961) found that nonionic macromolecules such as Tween 80 did not interfere with the preservative action of sorbic acid at the concentrations in which each is usually used (0.2% or less sorbic acid and 5% or less Tween 80). As a matter of fact, it is perhaps surprising that lecithin and Tween 80 do not interfere significantly with the action of polymyxin, since polymyxin behaves much like a quaternary ammonium cationic detergent (Jawetz, 1956).

In the laboratory trials, sorbic acid seemed very promising since, when added to the polymyxin media (50 or 75 μg/ml) in a concentration of 0.1%, it showed no inhibition of *S. aureus* and helped reduce the number of coagulase-negative cocci and *Bacillus* species. Other media could be eliminated from consideration rather readily because of poor activity against *S. epidermidis* and micrococci or inhibition of *S. aureus*. However, definitive field trials will have to be carried out before the addition of sorbic acid to the polymyxin medium can be generally recommended, since the preliminary field trials suggested the possibility of inhibition of *S. aureus*. Maltman, Orr, and Hinton (1960) have pointed out that staphylococci exposed to air drying (as they would be when free in the hospital environment) are more susceptible to certain deleterious influences. Studies in our laboratory

(Finegold, Sweeney, and Goldberg, *unpublished data)* have shown that, although the polymyxin medium with 75 μg/ml works well for most environmental cultures, it is definitely inhibitory for air-borne *S. aureus* (which would be exposed to even more drying) and that a polymyxin concentration of 25 μg/ml is much more suitable for this type of culture.

## Literature Cited

Blang, S. M., and S. S. Ahsan. 1961. Interaction of sorbic acid with nonionic macromolecules. J. Pharmacol. Sci. **50**:138-141.

Dye, W. E. 1959. Characteristics of some small colony-forming variants of *Staphylococcus aureus*. Ph.D. Thesis, University of Colorado School of Medicine, Denver.

Erlandson, A. L., Jr., and C. A. Lawrence. 1953. Inactivating medium for hexachlorophene (G-11) types of compounds and some substituted phenolic disinfectants. Science **118**:274-276.

Finegold, S. M., and E. E. Sweeney. 1961*a*. New selective and differential medium for coagulase-positive staphylococci allowing rapid growth and strain differentiation. J. Bacteriol. **81**: 636-641.

Finegold, S. M. and E. E. Sweeney. 1961*b*. An improved polymyxin medium for isolation and identification of *Staphylococcus aureus*. Bacteriol. Proc., p. 114.

Jawetz, E. 1956. Polymyxin, neomycin, bacitracin. Medical Encyclopedia, Inc., New York. Antibiotics Monograph no. 5.

Maltman, J. R., J. H. Orr, and N. A. Hinton. 1960. The effect of desiccation on *Staphylococcus pyogenes* with special reference to implications concerning virulence. Am. J. Hyg. **72**:335-342.

Quisno, R., I. W. Gibby, and M. J. Foter. 1946. A neutralizing medium for evaluating the germicidal potency of the quaternary ammonium salts. Am. J. Pharm. **118**:320-323.

Raj, H., and J. Liston. 1961. Detection and enumeration of coagulase-positive staphylococci. Bacteriol. Proc., p. 68.

Steers, E., E. L. Foltz, and B. S. Graves. 1959. An inocula replicating apparatus for routine testing of bacterial susceptibility to antibiotics. Antibiotics & Chemotherapy **9**:307-311.

Stuart, R. D. 1959. Transport medium for specimens in public health bacteriology. Public Health Repts. **74**:431-438.

# SENSITIVITY OF FUNGI TO ANTIMYCOTIC AGENTS

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In recent years the medical study of fungi has been intensified, not only of those that produce the more severe infections, but also of the saprophytic group. The latter group's importance, if such a term can be applied, stems from their widespread presence as common contaminants of culture media.

Clinical papers on mycotic infections are becoming more numerous and appear to parallel the introduction of newer antimycotic agents and drugs. The ever-increasing number of patients with infections attributable to fungi is perhaps due to a more thorough investigation of such cases as a result of the use (more often, the abuse) of antibiotics in bacterial infections. Antibiotics frequently upset the microbial equilibrium, and in this manner allow the proliferation of molds. We have observed marked exacerbations of dermatomycoses after penicillin therapy in patients with minimal interdigital lesions. Intestinal and pulmonary moniliasis has occurred after prolonged use of other antibiotics.

The study of fungi has become routine in our Allergy Section. This involves the isolation of such organisms from skin lesions, naso-pharyngo-bronchial exudate obtained by bronchial aspiration, fecal samples, and urine; these procedures are done concomitantly with bacterial and parasitic investigation of the patient. We also carry out the usual intradermal skin tests to determine and evaluate the individual's sensitivity to the antimycotic agent.

After establishing the pathogenicity or allergenicity of a fungus by tests based on local or systemic reactions to testing agents and other pertinent clinical data, we are then faced with the problem of eradicating the fungi in the affected individual. This should be done in the same way as in infections of bacterial origin. That is to say, the antimycotic should be selected, if possible, by sensitivity determinations similar to the "antibiograms" used in antibiotic assays. Our original studies began, therefore, with comparable techniques employing (i) various agents which either empirically or by reason of their mention in the scanty literature available on this subject were believed to have fungicidal or fungistatic properties and (ii) those

antimycotic medications which were then available commercially (Table 1). Subsequently, we limited our studies to those antifungal agents which we believed to be most effective.

TABLE 1. *Agents tested for antifungal activity*

| Commonly-used disinfectants | Agents with possible or known antifungal activity | Antifungal antibiotics |
|---|---|---|
| Alcohol, 60% | Phenyl mercuric chloride | Nystatin |
| Alcohol, 60%, plus ethyl acetate | Sodium borate | Griseofulvin |
| Potassium permanganate | Borogallic acid ester | Kabimycin (trichomycin) |
| Lugol's solution | Dodecyl cupric ammonium chloride | Amphotericin B |
| Thymol | Methyl-*p*-hydroxybenzoate | Colistin |
| Merthiolate | Propyl-*p*-hydroxybenzoate | |
| | Undecylenic acid | |
| | Undecylenic acid, mono and dioleate forms | |
| | Brilliant green | |
| | Gentian violet | |
| | Fuchsin, eosin | |
| | Dequadin chloride | |
| | Chlordantoin | |

In our first experiments to prove the efficacy of the methods described below, we employed various antimycotics on strains of the following species: *Torulopsis pulcherrima, Candida albicans, Epidermophyton floccusum, Trichophyton mentagrophytes, Aspergillus flavus, A. niger, Penicillium roquefortii, P. chrysogenum, Rhizopus nigricans, Hormodendrum* sp., *Geotrichum candidum,* and *Rhodotorula colostri.*

In these studies we sought therapeutic applications; for this reason, we used only those antifungal agents in general use. In their selection we took into account the sites of the fungi, the portals of entry, and the method of treatment (topical, nebulization, oral, or parenteral). We employed only those strains to which could be attributed a pathogenic or allergic role. We purposely eliminated some antimycotics because of their weak activity or because they were too impractical.

## Materials and Methods

For sensitivity determinations, we used the disc method on solid media, and liquid media in appropriate test dilutions. In general, the drug solutions used were calibrated so that one 0.05-ml drop produced the same concentration in 5 ml of liquid media as the weight of pure drug in the treated discs.

After finding a sufficiently high concentration of the antifungal agent to produce inhibition, we then attempted to find the minimal inhibitory concentration. Volatile substances, such as thymol, were limited entirely to liquid media after our first experiments.

The culture media used were the following: Sabouraud's solution (buffered to a pH of 7 and in some instances containing added vitamins), must agar, Czapek's medium, and occasionally blood agar.

The inoculation was done by means of drops of a fungus suspension equivalent in concentration to no. 1 on McFarland's scale for the yeast-like forms and to no. 2 or 3 on this same scale for the mycelial forms (depending on the degree of sporulation). On solid media, two drops of suspension were streaked out with Drigalsky's spatula, this being more convenient than the use of liquefied solid media (shake plates). The dish was placed face down in an incubator at 37 C for a period of 30 to 45 min to eliminate any excessive moisture; the treated discs, approximately 1 cm in diameter, were then placed on the surface. The discs were cut out from blotter paper with good absorptive quality. Several different colors were used, each color pertaining to a specific antimycotic agent. This detail however, could be disregarded if the discs were used in a particular order. Controlled tests with untreated discs and liquid media were done to determine if these had any inhibitive qualities of their own.

## Results

Results of the first series of experiments are summarized in Table 2. In this table some differences are noted in the results obtained in solid and liquid media with the same antifungal agent. This is due to the fact that the concentrations used were very low, 50 $\mu$g per disc and 10 $\mu$g per ml of medium (or the equivalent of these concentrations in units) for the majority of the compounds, except in those cases where large quantities could be employed. Amongst the latter can be listed sodium borate, where a concentration of 2,500 $\mu$g per disc or 500 $\mu$g per ml was employed, or boro-oxybenzoic acid ester or dodecyl cupric ammonium sulfate. In the latter case, the drug was used in saturated form, inasmuch as its solubility is within the limits of the other compounds.

We have noted that the action of some coloring agents is comparable to that of specific antimycotic agents. Mercury compounds are almost complete inhibitors.

The results given do not represent specific values; as will be shown in the second series of experiments, there are notable variations in the sensitivity of various strains of the same species to the antimycotic agent in vitro.

TABLE 2. *Inhibitory effect of various agents on certain fungi**

| Agent | Tri | | Epi | | Tor | | Geo | | Rhiz | | Asp | | Rho | | Can N | | Can E | | Horm | | Pen | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | L | D | L | D | L | D | L | D | L | D | L | D | L | D | L | D | L | D | L | D | L | D |
| *p*-Hydroxybenzoate | ± | − | − | − | − | − | − | − | − | − | − | − | − | − | ± | ± | − | − | − | − | − | − |
| Undecylenic acid | − | − | − | − | − | − | − | − | − | − | − | − | − | + | − | ± | − | − | + | + | − | − |
| Trichomycin | − | − | − | − | − | − | − | − | − | − | − | − | − | − | − | − | ± | + | − | − | + | + |
| Griseofulvin | − | − | − | − | + | ± | − | − | − | − | − | − | − | − | − | − | ± | ± | − | − | − | − |
| Nystatin | − | − | − | ± | ± | + | − | + | + | + | − | + | + | + | + | + | − | + | − | − | ± | + |
| Lugol's solution | − | − | − | − | − | − | − | − | + | − | + | − | − | − | − | − | + | + | + | + | − | − |
| Potassium permanganate | − | − | − | − | − | − | − | − | ± | ± | − | − | − | − | ± | ± | − | − | − | − | − | − |
| Sodium borate | − | − | − | − | − | ± | − | + | − | − | − | + | − | − | + | + | − | − | + | + | − | − |
| Thymol | + | | − | | + | | − | | + | | + | | + | | + | | + | | + | | + | |
| Fuchsin | + | ± | − | − | − | + | − | − | + | + | − | − | − | − | − | − | + | ± | + | + | − | − |
| Brilliant green | + | + | ± | + | + | + | + | ± | + | + | + | − | − | − | − | − | − | − | + | + | − | − |
| Gentian violet | ± | + | ± | − | + | + | + | − | + | + | + | + | − | − | + | + | + | + | + | + | + | + |
| Dodecyl cupric $(NH_4)_2SO_4$ | − | − | − | + | − | − | − | − | ± | + | − | + | − | − | − | − | + | + | − | − | − | − |
| Phenyl mercuric chloride | + | + | + | + | + | + | + | + | + | + | + | ++ | + | + | + | + | + | + | + | + | + | + |
| Boro-oxybenzoic ester, 5% | − | − | − | + | − | − | − | − | − | − | − | + | − | − | − | − | − | − | − | − | − | − |

*Abbreviations used: Tri = *Trichophyton mentagrophytes;* Epi = *Epidermophyton floccosum;* Tor = *Cryptococcus neoformans (Torula);* Geo = *Geotrichum candidum;* Rhiz = *Rhizopus nigricans;* Asp = *Aspergillus flavus;* Rho = *Rhodotorula colostri;* Can N = *Candida albicans* N; Can E = *C. albicans* 365; Horm = *Hormodendrum* sp.; Pen = *Penicillium roquefortii;* L = assay by the tube dilution method with liquid media; D = assay by the disc method. All agents were tested with 50 μg discs and 10 μg/ml solutions, except sodium borate and boro-oxybenzoic ester (2,500 μg disc and 500 μg/ml), nystatin (200 μg disc and 40 μg/ml), and dodecyl cupric ammonium sulfate (saturated solution).

In the case of griseofulvin, it is known that its fungistatic action has a pattern all of its own. In this instance, because the zones of inhibition were small or not produced at all with the concentrations used by us, we were able to observe microscopically the toxic effect known as the "curling factor."

Following the same procedure we studied five additional antimycotic agents: amphotericin B, clordantoin (Lapan, 1959), Dequadin (Babbs et al., 1956; Cox and Darcy, 1961), Totazina and colistin (Jörg, *personal communication).* In addition we observed in a few cases the effect of undecylenic acid as compared to its derivatives, the mono and dioleate forms.

In Table 3 are shown the results obtained with various strains of *Candida* isolated from our patients. The symbols +, ++, and +++ for solid media correspond to inhibition zones of less than 2 mm in diameter, to those of between 2 and 4 mm, and to those greater than 4 mm, respectively. It can be noted that undecylenic acid in a few cases exerts a very slight inhibition. Sodium borate at a very high concentration is somewhat more effective. The coloring agents show an irregular and variable action. There is one strain which is resistant to amphotericin B, but none which is resistant to nystatin, Dequadin chloride, clordantoin, or mercurials.

TABLE 3. *Effect of various antifungal agents on clinical isolates of Candida*

| Agent | *C. albicans* | | | | | | | | | | *C. tropicalis* | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | N | 365 | 370 | RGB | CB | 373 | 389 | 394 NF | 394 MF | 394 P | 400 P | 405 Pie |
| Undecylenic acid | + | + | – | – | – | – | – | + | – | – | – | – |
| Sodium borate | – | – | + | – | + | + | ++ | – | + | – | – | – |
| Trichomycin | – | – | – | ++ | + | – | + | – | + | – | – | – |
| Gentian violet | +++ | ++ | +++ | ++ | + | ++ | +++ | – | + | – | – | + |
| Brilliant green | + | + | +++ | + | – | + | +++ | ++ | +++ | – | – | ++ |
| Nystatin | +++ | ++ | + | + | ++ | + | +++ | + | +++ | + | + | +++ |
| Amphotericin B | +++ | +++ | +++ | ++ | ++ | + | ++ | + | +++ | + | + | ++ |
| Clordantoina | ++ | ++ | ++ | ++ | ++ | + | + | +++ | +++ | – | – | +++ |
| Dequadin | ++ | ++ | + | ++ | ++ | ++ | + | ++ | ++ | + | + | ++ |
| Griseofulvin | – | – | – | – | – | – | – | – | + | – | – | + |
| Colistin | – | – | + | – | – | – | + | ± | – | + | + | + |
| Totazina | – | – | – | – | – | – | – | – | – | ± | ± | ± |
| Mercurials | +++ | +++ | +++ | +++ | +++ | | | | | ++ | +++ | +++ |

We completed 18 additional assays on different strains of *C. albicans* isolated from our patients; the results obtained are not enumerated, inasmuch as these determinations were not sufficiently large in number for us to determine which compounds are preferable and which should be discarded.

In Table 4 we depict the results obtained with *Trichophyton*, *Aspergillus*, and other fungi isolated from cutaneous and ear infections.

TABLE 4. *Effect of various antifungal agents on fungi isolated from cutaneous and ear infections*

| Agent | *Epidermophyton floccosum* 395 | *Trichophyton mentagrophytes* 381 | *Trichophyton menagrophytes* 401 | *Aspergillus flavus* 398 | *Aspergillus niger* 407 |
|---|---|---|---|---|---|
| Undecylenic | + | – | – | – | – |
| Sodium borate | – | + | – | – | – |
| Trichomycin | – | + | ++ | + | – |
| Gentian violet | + | – | ++ | + | – |
| Brilliant green | – | + | + | + | + |
| Nystatin | +++ | +++ | +++ | + | + |
| Amphotericin B | – | ++ | ++ | – | – |
| Clordantoina | +++ | + | – | +++ | + |
| Dequadin | – | – | +++ | ++ | ++ |
| Griseofulvin | – | + | – | – | – |
| Colistin | – | – | – | – | – |
| Totazina | – | + | – | – | – |
| Mercuriates (0.1 to 10 μg/ml) | +++ | ++ | ++ | ± | +++ |

*Comparative quantitative studies.* We tried to determine in vitro the minimal concentrations of each antimycotic required for each species isolated. The object of this was to try to establish certain principles of dosage. These data, which will include determinations of blood levels and urine levels, will be described in a subsequent publication.

*Antimycotic synergism.* In some cases, we observed synergism between two antimycotics: e.g., clordantoin and nystatin, and sodium borate and nystatin. We have begun additional studies with respect to this phenomenon.

## Literature Cited

Babbs, M., H. O. J. Collier, W. C. Austin, M. D. Potter, and E. P. Taylor. 1956. Salts of decamethylene-bis-4-aminoquinaldinium (Dequadin). A new antimicrobial agent. J. Pharm. Pharmacol. 8:110-119.

Cox, W. A., and P. F. Darcy. 1961. The activity of polymenlene-bis-4-aminoquinaldinium salts against Pityrosporum ovale and Candida albicans. J. Pharm. Pharmacol. 13:34-38.

Lapan, B. 1959. A new agent for the treatment of vaginal candidiasis. Am. J. Obstet. Gynecol. 78: 1320.

# CLINICAL USE OF THE DISC SENSITIVITY TEST

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The dětermination of the sensitivity of microorganisms to chemotherapeutics in vitro is of great value in selecting proper agents for the specific therapy of patients with bacterial infections. In particular, the disc method, because of its simplicity, is widely used in many laboratories. Since Morley (1945) and Bondi et al. (1947) reported this technique, a large number of studies have been concerned with this procedure. Test conditions and interpretation of results, however, differ among various workers and laboratories. Branch (1955) and Branch et al. (1959) advocated international standardization of the test. I have studied the fundamental conditions and clinical application of the sensitivity disc method since 1954. In this paper I wish to summarize my work, in the hope that it may contribute to the standardization of the method.

## Experimental

The results of the sensitivity-disc method are supposed to be influenced by various experimental conditions. As for the medium, such factors as pH and thickness of the agar medium, concentration of NaCl, content and diffusiveness of the agar, and the effect of adding blood or glucose were studied. As for the agents, their diffusion in the medium, deterioration of potency during incubation, and survival potency of the disc during storage were studied. At first, a three-disc method in which sensitivity was graded according to the presence or absence of an inhibition zone was employed. In this method, the concentration of the agent in the agar layer immediately around the disc is not equal to the content of the disc (Fig. 1); it varies with the type of agent and the initial content of the disc. For example, the minimal inhibitory concentration (MIC) of furazolidone, determined by the agar dilution method, is markedly different from the content of discs which show a zone of inhibition (Fig. 2).

In the three-disc method, a low-content disc must be prepared. Low-content discs, especially of penicillin, are less stable during storage than are high-content discs (Fig. 3).

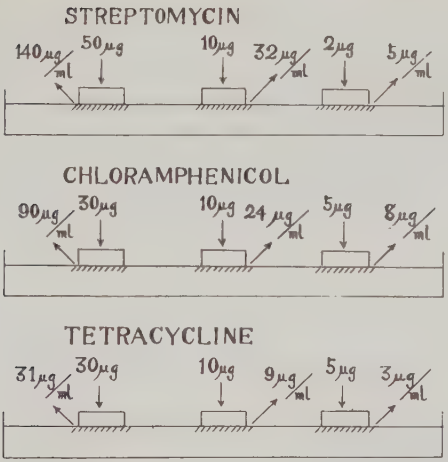
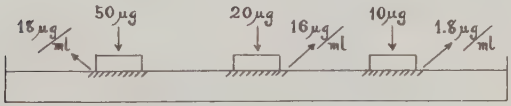



FIG. 1. *Relationship between the initial drug content of discs (6mm in diameter) and the drug concentration around the disc after 6 hr absorption time.*




| INITIAL CONTENT OF DISC | 50μg | 20μg | 10μg |
|---|---|---|---|
| M.I.C. TO THE ORGANISMS WHICH GROW INHIBITION ZONE | < 25μg/ml | < 25μg/ml | < 2.5μg/ml |

FIG. 2. *Relationship of the initial content of furazolidone to the concentration around the disc after 6 hr and to the MIC of the organisms (31 strains).*

From the results of these experiments with the three-disc method, it is difficult to establish, at the present time, a definite relationship between drug content and interpretation of sensitivity. Accordingly, I studied the single-disc method, which is thought to be useful for obtaining consistent values and which uses the relatively stable high-content disc.

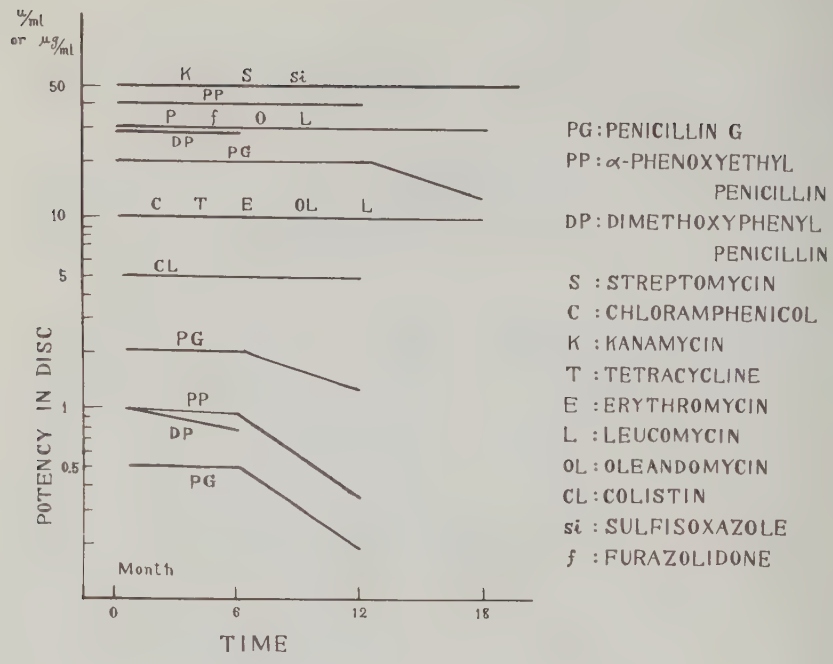



FIG. 3. *Stability of discs during storage at 4 C with drierite.*

*Single-disc method.* The following modified Mueller-Hinton medium, with or without 5% human blood, is used: heart infusion, 300 ml; Casamino acid, 16.5 g; starch, 1.5 g; glucose, 2 g; L-tryptophan, 0.05 g; L-cystine, 0.05 g; biotin, 0.000005 g; agar ("NISSAN"), 15 g; and distilled water to 1,000 ml. The pH after sterilization is 7.3 to 7.4. The NaCl concentration is 0.7 ± 0.2%. Agar is controlled for constancy of diffusiveness by using dye diffusion as an indicator.

To make the measurements accurate, the drug content of the discs is determined (Table 1) under such conditions as to: (i) produce an inhibition zone at the lower limit of clinical resistance; (ii) provide a linear relation between the diameter of the inhibition zone and the logarithmic value of the concentration; and (iii) contain enough drug to retain the initial potency for at least 1 year.

The agar medium, melted and cooled to about 50 C, is poured into a flat-bottom plate placed on a level surface. The thickness of 20 ml of medium in a petri dish with an inner diameter of 85 mm was 3.4 to 3.5 mm. If dishes of other sizes were used, the amount of medium was changed so as to provide the same thickness. One

loopful of a bacterial colony (approximately 2 mg) is suspended homogeneously in 5 ml of sterile physiological saline. One drop (approximately 0.025 ml) of the bacterial suspension is placed on a plate and spread uniformly by rolling with about ten glass beads (diameter of 4 to 6 mm). The inoculum in this case is approximately $5 \times 10^4$ to $10 \times 10^4$ cells per $cm^2$. A uniform inoculum can also be obtained by flooding the plate with a bacterial suspension and removing the excess with a pipette. After the application of the disc, the plates are kept at 5 to 15 C for 4 hr. Then they are incubated at 37 C for 16 to 20 hr.

TABLE 1. *Drug content of discs**

| Chemotherapeutic agent | Content of disc |
|---|---|
| | μg |
| Penicillin G | 20 † (potency) |
| α-Phenoxyethyl penicillin | 40 † (potency)<br>L-Form 62.5% |
| Dimethoxyphenyl penicillin | 30 (potency) |
| Streptomycin | 50 (potency) |
| Chloramphenicol | 100 (potency) |
| Tetracycline | 200 (potency) |
| Oxytetracycline | 200 (potency) |
| Erythromycin | 50 (potency) |
| Kanamycin | 50 (potency) |
| Oleandomycin | 30 (potency) |
| Leucomycin | 30 (potency) |
| Colistin | 5 (potency) |
| Novobiocin | 20 (potency) |
| Mikamycin | 30 (potency)<br>A 26.5 (potency)<br>B 3.5 (potency) |
| Fradiomycin | 20 (potency) |
| Sulfisoxazole | 400 |
| Sulfisomidine | 400 |
| Sulfamethoxypyridazine | 400 |
| Sulfisomezole | 400 |
| Sulfamethizole | 400 |
| Sulfadimethoxine | 400 |
| Sulfaphenazole | 400 |
| Furazolidone | 20 |
| Nitrofurantoin | 100 |

*Disc: 8mm in diameter; water absorption: 0.027 ± 0.004 ml.

†Units instead of μg.

To calculate the sensitivity, the average diameter of the inhibition zone is measured. Then the MIC is calculated by means of standard curves representing the relation between the size of the inhibition zone and the MIC (Fig. 4). The standard curves in the diagram were constructed on the basis of experiments repeated more than seven times with approximately 84 strains of test organisms (Table 2).

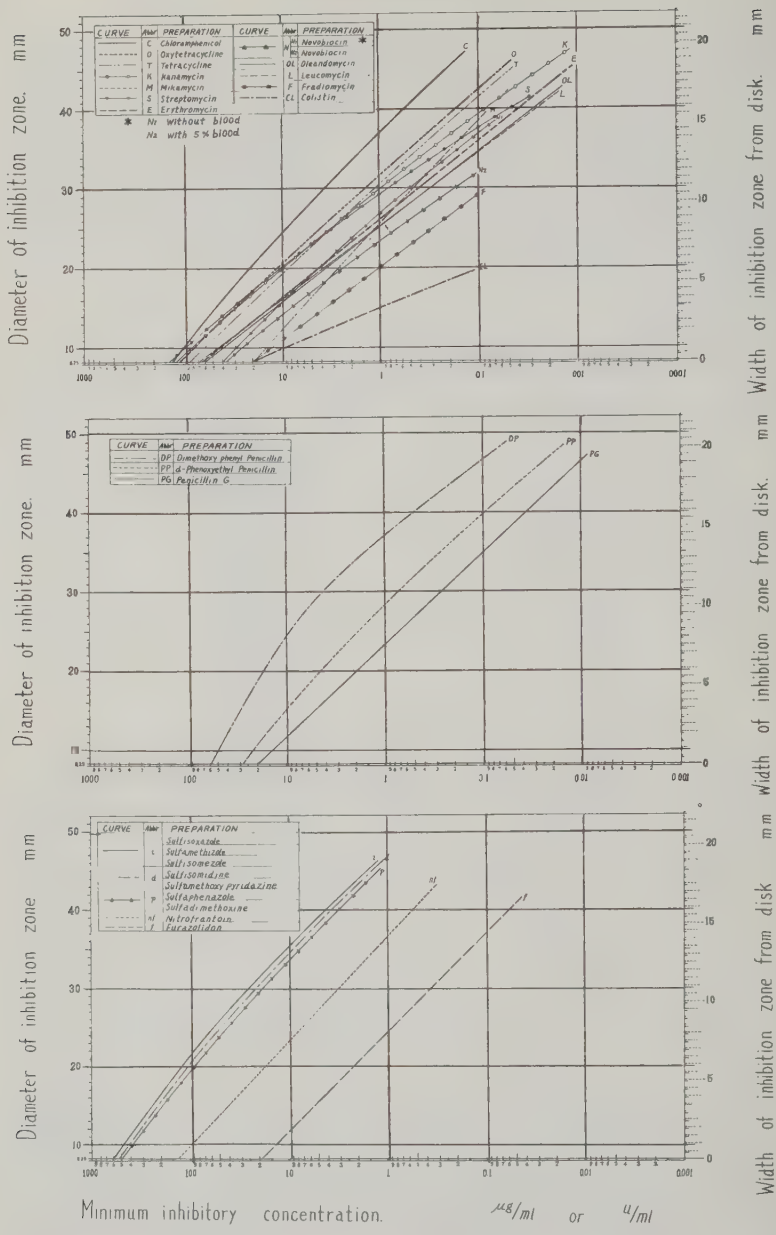



FIG. 4. *Standard curves representing the relationship between the size of the zone of inhibition and the MIC.*

TABLE 2. *Organisms used in the experiment for constructing standard curves*

| Organism | No. of strains |
|---|---|
| *Staphylococcus aureus* | 32 |
| *Streptococcus hemolyticus* | 3 |
| Viridans streptococci | 3 |
| *Neisseria gonorrhoeae* | 2 |
| *Diplococcus pneumoniae* | 5 |
| *Corynebacterium diphtheriae* | 2 |
| *Haemophilus influenzae* | 1 |
| *Escherichia coli* | 12 |
| *Pseudomonas aeruginosa* | 6 |
| *Klebsiella pneumoniae* | 5 |
| *Salmonella paratyphi* A | 2 |
| *Salmonella paratyphi* B | 1 |
| *Shigella flexneri* | 5 |
| *Shigella sonnei* | 3 |
| *Morganella* | 2 |
| Total | 84 |

Then, to establish the range of experimental error of the disc method, the deviation from the standard curve, with 20 strains for each drug, each of whose MIC is relatively strictly determined, is calculated in terms of rejection limit. Rejection limit was calculated from the formula: $X \pm \sigma t_{0.05} \sqrt{\frac{n+1}{n}}$, where X stands for the MIC corresponding to the size of the inhibition zone on the standard curve, n for the number of strains tested, t for t-distribution of Student, d for deviation from the standard curve, and $\sigma$ for the sample standard

TABLE 3. *Range of deviation from the standard curve with the disc method**

| Chemotherapeutic agent | Rejection limit |
|---|---|
| Penicillin G | 3.2-0.31 |
| Streptomycin | 3.2-0.31 |
| Chloramphenicol | 2.6-0.39 |
| Tetracycline | 2.6-0.38 |
| Erythromycin | 2.3-0.43 |
| Oleandomycin | 2.6-0.38 |
| Kanamycin | 2.9-0.35 |
| Novobiocin | 2.2-0.31 |
| α-Phenoxyethyl penicillin | 3.0-0.33 |
| Dimethoxyphenyl penicillin | 3.1-0.32 |
| Colistin | 3.6-0.28 |
| Sulfisoxasole | 3.4-0.29 |
| Furazolidone | 2.6-0.39 |

*Where MIC corresponding to the diameter of the zone of inhibition is taken as 1.0; $\alpha = 0.05$; $n = 20$.

deviation obtained from the formula: $\sqrt{\frac{\Sigma d^2}{n-1}}$ (Table 3). For reference, the range of the MIC obtained by the twofold agar dilution method, which is believed to yield reproducible results, is shown in Table 4.

TABLE 4. *Range of MIC values obtained by the two-fold agar dilution method**

| Chemotherapeutic agent | Rejection limit |
|---|---|
| Penicillin G | 2.7-0.37 |
| Phenoxyethyl penicillin | 2.7-0.38 |
| Dimethoxyphenyl penicillin | 3.1-0.32 |
| Streptomycin† | 2.2-0.45 |
| Chloramphenicol† | 2.5-0.40 |
| Tetracycline | 2.7-0.38 |
| Erythromycin | 2.2-0.46 |
| Oleandomycin | 2.8-0.37 |
| Kanamycin† | 2.3-0.44 |
| Novobiocin | 2.7-0.37 |
| Colistin† | 2.7-0.38 |
| Sulfisoxazole† | 3.0-0.33 |
| Furazolidone | 2.2-0.45 |

*Where sample mean is taken as 1.0; $\alpha = 0.05$; n = 12 to 14. Test organism: *Sta* FDA209P.
†Test organism: *E. coli* NIHJ.

*Application and modification of the disc method.* 1) Radial streak method:—From the zone immediately surrounding a disc left on the center of a plate for 4 hr, several types of bacterial suspension are radially and simultaneously stroked. After incubation, inhibition width from the circumference of the disc can be translated into MIC by means of the above-mentioned standard curve. With this method, it is adequate to streak one loopful of about seven to ten times diluted bacterial suspension to make the inoculum size per $cm^2$ equivalent to the usual method.

2) Test of bactericidal action:—The original plate in which the disc test took place is replicated, by means of a velvet pad or other material, to another plate. Bactericidal action can be estimated by reference to the growth of organisms in the latter plate.

3) Test of combined action:—The disc method in which two agents diffuse from separate adjoining discs sometimes produces an inhibition zone, the form of which may indicate synergism, antagonism, or indifference. However, the fact that the diffusion of agents in the agar may occasionally influence each other must be taken into consideration.

4) Disc method with penicillinase-producing staphylococci:—By use of the penicillin disc method with penicillinase-producing staphylococci, the inhibition zone is liable to become irregular and smaller

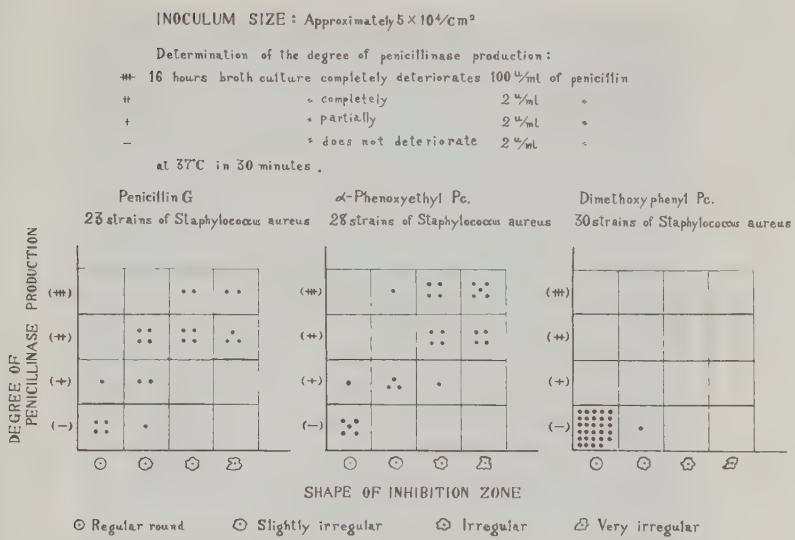

INOCULUM SIZE : Approximately 5 × 10⁴/cm²
Determination of the degree of penicillinase production :
(+++) 16 hours broth culture completely deteriorates 100 u/ml of penicillin
(++) " completely 2 u/ml "
(+) " partially 2 u/ml "
(−) " does not deteriorate 2 u/ml "
at 37°C in 30 minutes.
Penicillin G
23 strains of Staphylococcus aureus
α-Phenoxyethyl Pc.
28 strains of Staphylococcus aureus
Dimethoxyphenyl Pc.
30 strains of Staphylococcus aureus
DEGREE OF PENICILLINASE PRODUCTION
(+++)
(++)
(+)
(−)
SHAPE OF INHIBITION ZONE
Regular round
Slightly irregular
Irregular
Very irregular

FIG. 5. *Relationship between penicillinase production and the shape of the zone of inhibition after incubation for 12 hr.*

with the lapse of time (Fig. 5). The degree of irregularity of the inhibition zone correlates relatively well with the penicillinase-producing ability of the organisms tested under the same condition. MIC determined by the radial streak method, however, is almost equal to the one obtained by the agar dilution method under the same conditions (Fig. 6).

5) Penicillin and sulfonamide disc method:—In penicillin or sulfonamide therapy, though the causative organisms belong to the resistant range according to the sensitivity test, the drug is often clinically effective. Therefore, the influence of the inoculum on the results with penicillin or sulfonamide discs, and their correlation with the clinical response to the drug, have been studied. The penicillin disc test, with a $10^{-2}$ to $10^{-1}$ inoculum (i.e., approximately $10^3$ to $10^4/cm^2$), and the radial streak disc test seemed to have only slightly better correlation with the clinical responses, compared to the test with the usual inoculum (i.e., approximately $10^5/cm^2$) and with $10^{-3}$ inoculum (i.e., $10^2/cm^2$) (Fig. 7). And the sulfonamide disc method with $10^{-1}$ to $10^{-3}$ inoculum (i.e., approximately $10^2$ to $10^4/cm^2$) or radial streak inoculation had a better correlation with the clinical responses than did the usual inoculum (i.e., $10^5/cm^2$) (Fig. 8).

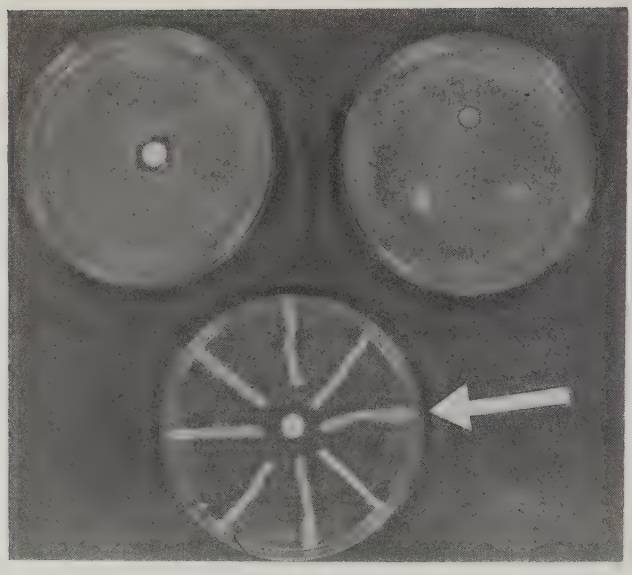


FIG. 6. *Penicillin disc methods used to test a penicillinase-producing strain of Staphylococcus aureus (MIC of 0.6 units/ml by the agar dilution method). The single-disc (20 units of penicillin G) method given an irregular zone of inhibition. The three-disc (10, 2, and 0.5 units of penicillin G) method does not show any clear zones of inhibition. The radial streak method shows zones of inhibition corresponding to the MIC obtained by the dilution method.*

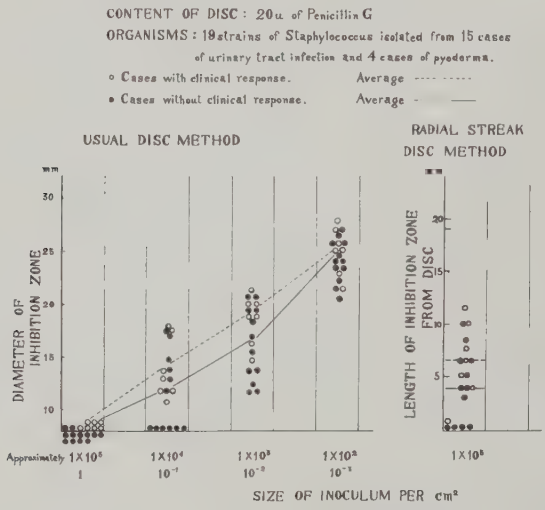



FIG. 7. *Influence of inoculum size on the size of the zone of inhibition and on the clinical response of a penicillin-resistant Staphylococcus.*

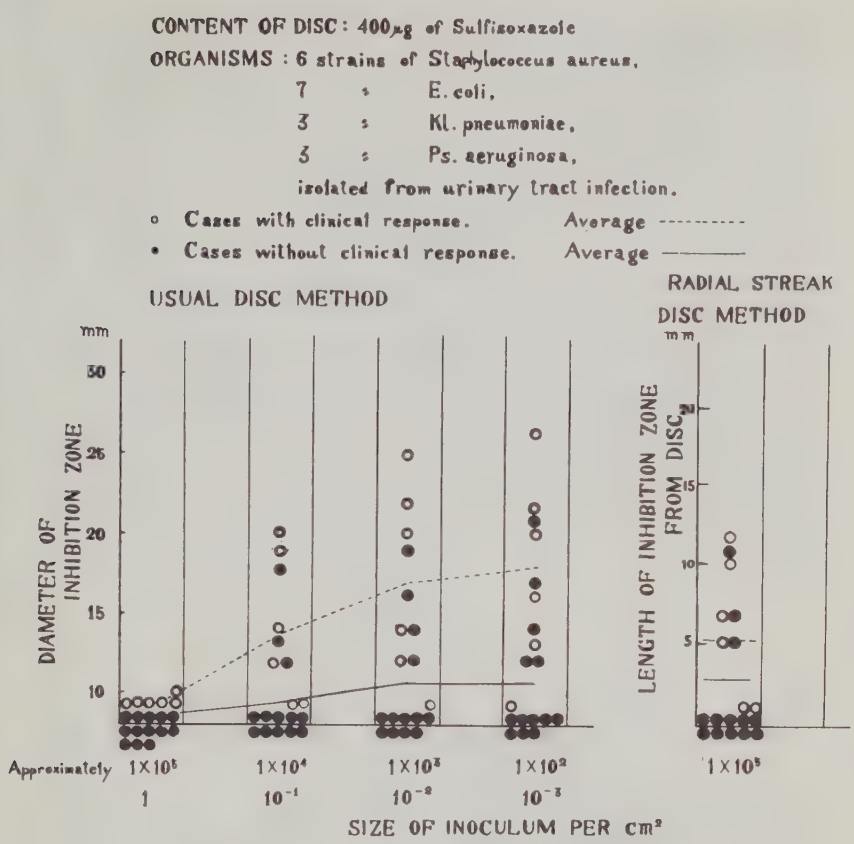



FIG. 8. *Influence of inoculum size on the size of the zone of inhibition and on the clinical response of sulfonamide-resistant organisms.*

6) Simple method:—Sensitivity determined by the disc method, omitting the 4-hr prediffusion period, is still useful for clinical purposes. Sensitivity of *Staphylococcus, Escherichia, Klebsiella, Shigella, Pseudomonas,* and *Salmonella* to chemotherapeutic agents can be estimated by means of the standard curves (Fig. 9) drawn experimentally without prediffusion.

7) Rapid method:—Over a base layer of 16 ml of agar medium in a petri dish (inner diameter of approximately 85 mm), 4 ml of melted medium inoculated with one loopful (approximately 2 mg) of a bacterial colony is prepared and hardened. Immediately after the disc is placed on the plate, the plate is incubated at 37 C for 3 to

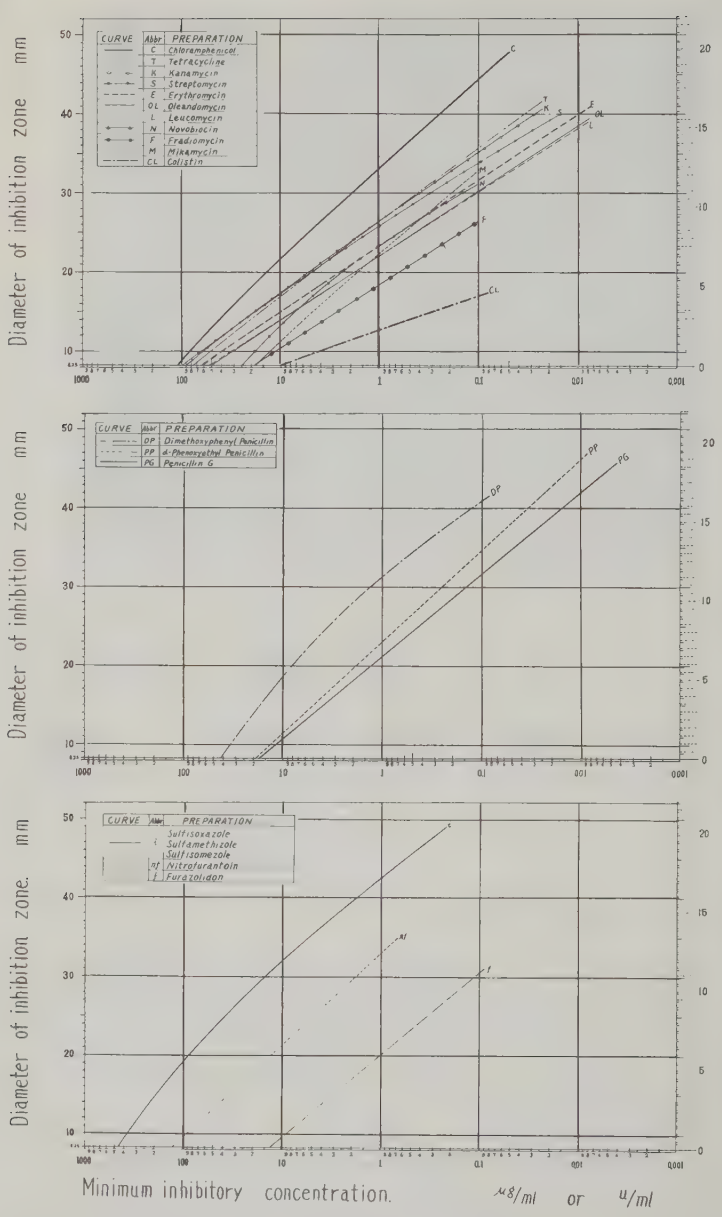



FIG. 9. *Standard curves representing the relationship between size of the zone of inhibition and the MIC without prediffusion.*

4 hr. As a screening test, the size of the inhibition zone can be translated to the sensitivity of the organisms in terms of MIC by reference to a diagram constructed experimentally (Fig. 10). With this method, however, the inoculum is so heavy that penicillinase-producing organisms are likely to be determined as strongly resistant to penicillin. The same relationship is observed for organisms in general with the sulfonamide disc method. Moreover, oxidation-reduction dyes, such as tetrazolium chloride or 2,6-dichlorophenolindophenol, are sometimes available to make the inhibition zone more distinct.

*Clinical evaluation of sensitivity.* The relationship between MIC determined by the above-mentioned disc method and clinical response has been investigated. Certain diseases and isolated causative organisms are shown in Table 5. In this investigation, cases in which the drug produced symptomatic or bacteriological response, even if transient, and cases with good response to chemotherapeutics given as supplemental treatment to surgical management, were all regarded as effective. In some disease conditions, the relationship between the MIC of the chemotherapeutics and the isolated organisms and the clinical responses have been investigated (Fig. 11). Additional data are summarized with respect to each chemotherapeutic agent used (Fig. 12 and 13).

TABLE 5. *Organisms isolated from 1,394 cases (913 patients)*

| Disease | No. of cases | Isolated organism | No. |
|---|---|---|---|
| Urinary tract infection | 303 | *Staphylococcus* | 813(58%) |
| Pulmonary abscess, bronchiectasis | 167 | Pneumococcus | 32 |
| Pneumonia | 67 | *Streptococcus hemolyticus* | 58 |
| Bronchitis | 57 | Viridans streptococci | 32 |
| Pharyngitis, tonsillitis, scarlet fever | 33 | *Streptococcus faecalis* | 5 |
| Pyothrax | 66 | Gram-positive cocci | 5 |
| Sepsis | 82 | Gram-positive diplococci | 8 |
| Subacute bacterial endocarditis | 32 | *Neisseria* | 6 |
| Meningitis | 11 | *Neisseria meningitidis* | 2 |
| *Salmonella* infection | 14 | Gram-negative cocci and diplococci | 28 |
| Bacillary dysentery | 50 | *Escherichia coli* | 203(15%) |
| Acute enteritis | 2 | *Klebsiella* | 21 |
| Abscess of liver, cholangitis, cholecystitis | 59 | *Salmonella* | 14 |
| Acute peritonitis | 28 | *Shigella* | 50 |
| Pyoderma, furuncle, carbuncle | 159 | *Proteus* | 16 |
| Abscess | 29 | *Pseudomonas* | 41 |
| Wound infection | 34 | *Alcaligenes* | 14 |
| Cellulitis | 6 | *Serratia* | 2 |
| Subungual infection | 4 | *Haemophilus influenzae* | 6 |
| Osteomyelitis | 25 | *Corynebacterium* | 4 |
| Postoperative wound infection | 60 | Gram-negative rods | 30 |
| Myositis | 23 | Gram-positive rods | 4 |
| Mastitis | 12 | | |
| Arthritis | 14 | | |
| Otitis media | 51 | | |
| Lymphadenitis | 6 | | |

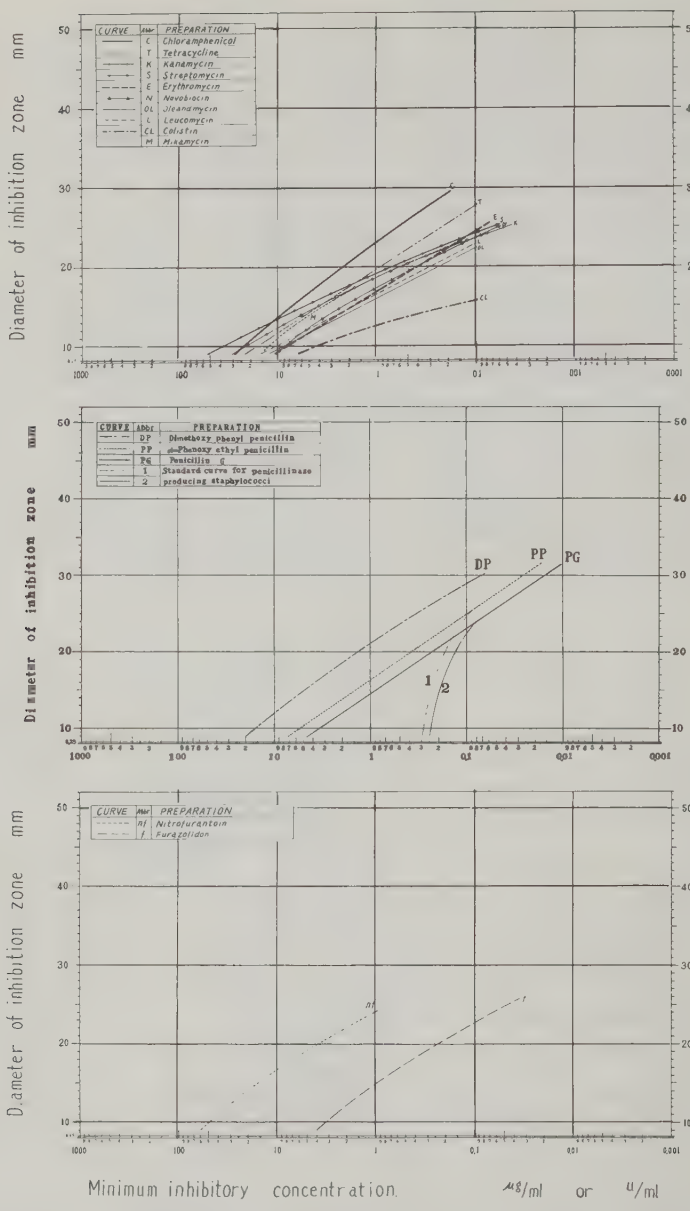



FIG. 10. *Standard curves representing the relationship between size of the zone of inhibition and the MIC by the rapid method.*

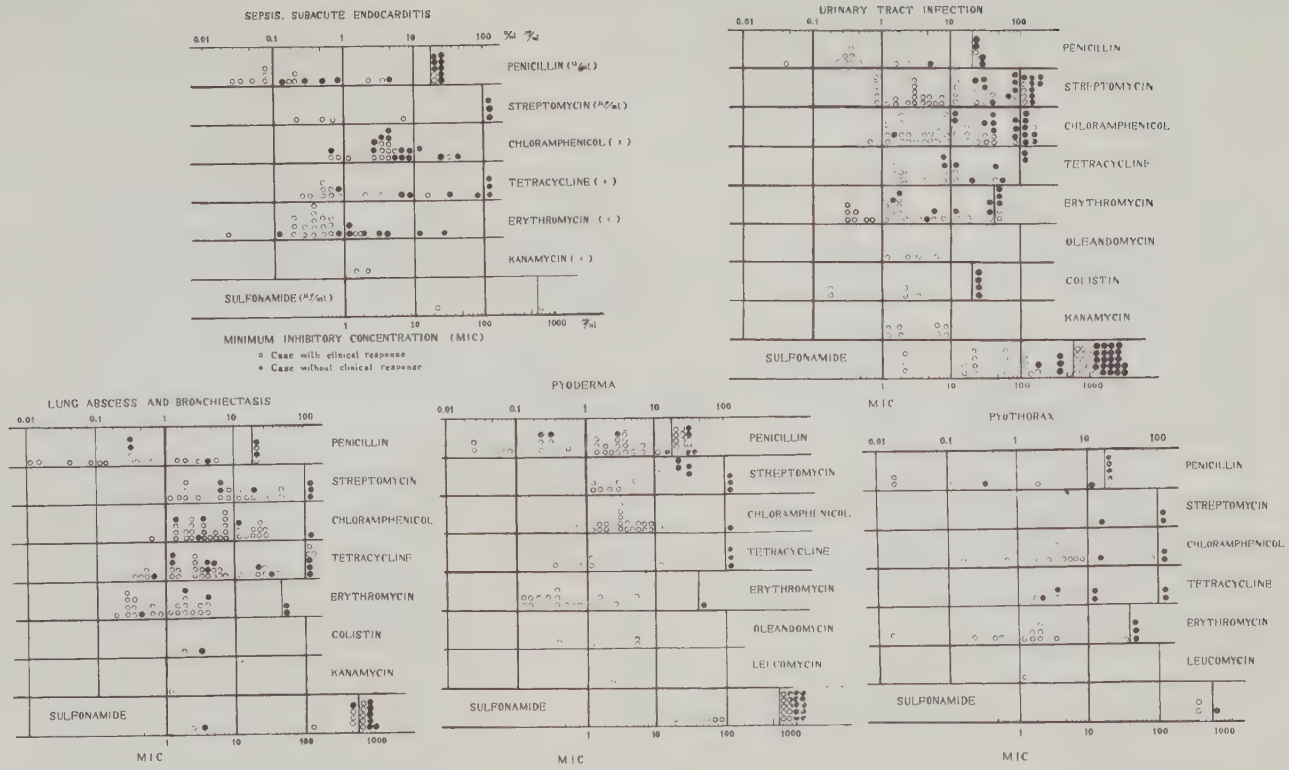



FIG. 11. *Relationship between MIC and clinical response, according to disease.*

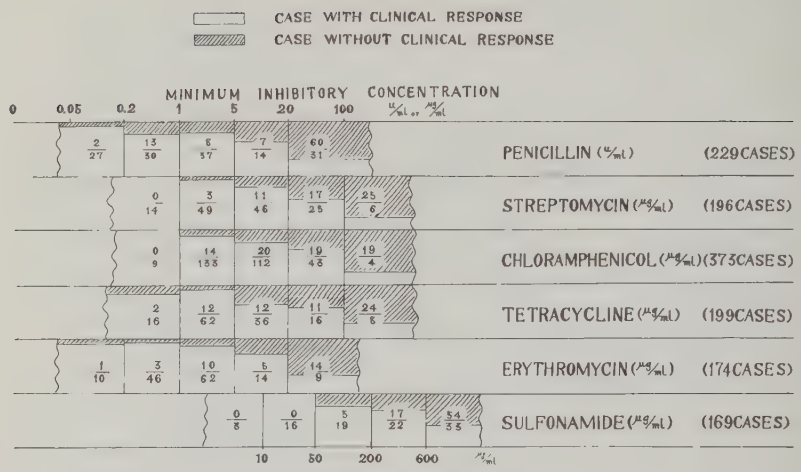



FIG. 12. *Relationship between sensitivity classified in terms of MIC to the ratio of clinical response.*

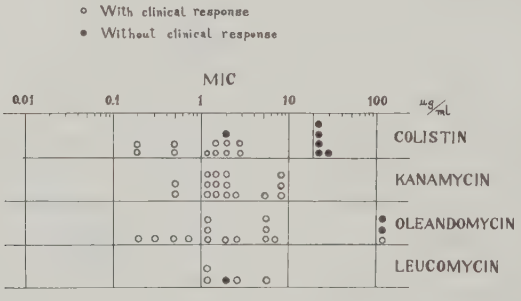



FIG. 13. *Relationship between MIC and clinical response to various antibiotics.*

To a certain extent, a continuous correlation was observed between the MIC and the clinical responses (Fig. 12). Consequently, sensitivity expressed in terms of MIC is thought to be of value for clinical use. On the basis of these results and assumed biologically active concentrations in body fluids, the borderline values of clinical sensitivity in terms of MIC (Table 6) seem to be reasonable at present. Some of these numerical values noted here are very close to ones already reported by Tunneval and Ericsson (1954).

TABLE 6. *Numerical value of clinical sensitivity in terms of MIC with reference to the localization of infection and the dosage or the route of administration**

| Classification | Very sensitive | Fairly sensitive | Slightly sensitive | Resistant |
|---|---|---|---|---|
| Clinical interpretation | Therapeutic effect expected with ordinary dosage, in generalized infection, respiratory tract infection, etc. | Therapeutic effect expected, with high blood concentration, in generalized infection, respiratory infection, etc. | Therapeutic effect expected, when high concentration of the drug is administered to the lesion of infection (e.g., urinary tract infection, topical use for wounds, etc.) | Therapeutic effect generally not expected, except topical use at high concentration. |
| Penicillin G † | < 0.2 | < 2 | < 20 | > 20 |
| Streptomycin | < 4 | <20 | <100 | >100 |
| Chloramphenicol | < 4 | <15 | < 60 | > 60 |
| Tetracycline | < 2 | <10 | < 60 | > 60 |
| Erythromycin | < 1 | < 5 | < 30 | > 30 |
| Sulfonamide | <20 | <100 | <500 | >500 |
| Kanamycin | <(4) | <(20) | <(100) | >(100) |
| Oleandomycin | <(1) | <(5) | <(30) | >(30) |
| Leucomycin | <(1) | <(5) | <(30) | >(30) |
| Colistin | <(1) | <(4) | <(20) | >(20) |
| Novobiocin | <(1) | <(4) | <(20) | >(20) |
| α-Phenoxyethyl penicillin† | <(0.2) | <(2) | <(20) | >(20) |
| Dimethoxyphenyl penicillin | <(0.5) | <(4) | <(40) | >(40) |
| Fradiomycin | <(1) | <(4) | <(20) | >(20) |
| Mikamycin | <(0.5) | <(4) | <(20) | >(20) |
| Furazolidone | <(1) | <(5) | <(20) | >(20) |
| Nitrofurantoin | <(5) | <(20) | <(100) | >(100) |

*Results are expressed as μg/ml. Figures in parentheses were tentatively decided on the basis of biologically active concentration in body fluid and the results of a few clinical cases.

†Units/ml.

## Discussion

Although sensitivity of microorganisms has been expressed customarily as "sensitive" or "resistant," internationally accepted MICs corresponding to the terms "sensitive" or "resistant" have never been established. Even if numerical values were tentatively decided, they would have to be altered according to improvements in drugs or in the method of administration. Consequently, the results of the sensitivity test in the clinical laboratory should be expressed in terms of MIC, with the terms "sensitive" and "resistant" as only a supplement. It is supposed to be reasonable for the clinician to select the drug on the basis of MIC determination in the laboratory, consulting various factors such as active concentration in body fluid or the lesion, pH of the lesion, mode of action of the agent (bacteriostatic or bactericidal), side effects of the agent, possible penicillinase production of the causative organisms, nature of the disease, and clinical experience in the past. Therefore, it seems useful to add to the sensitivity disc a table of probable active concentrations

(Kanazawa, 1956; Kanazawa and Kuramata, 1960; Miyamura and Kanazawa, 1951) of the drugs in the body fluid by various dosages and various routes of administration.

## Literature Cited

Bondi, A. S., E. H. Spaulding, D. E. Smith, and C. C. Diety. 1947. A routine method for the rapid determination of susceptibility to penicillin and other antibiotics. Am. J. Med. Sci. **213**: 221.

Branch, A. 1955. Experience with controlling antibiotic sensitivity tests in Department of Veterans Affairs Hospital Laboratories in Canada. Antibiotics Ann. 1954-1955, p. 1125.

Branch, A., D. H. Starkey, E. Edna, and B. A. Power. 1959. The present status of control of manufactured antibiotic sensitivity disc. Can. Med. Assoc. J. **81**:175.

Kanazawa, Y. 1956. Sensitivity disc method and active level of chemotherapeutic in body fluid as the guides for chemotherapy. Nihon Rinsho (Japanese Clinic) **14**:635.

Kanazawa, Y., and T. Kuramata. 1960. Assay method of biologically active sulfonamide in body fluid. Chemotherapy (Tokyo) **8**:478.

Miyamura, S., and Y. Kanazawa. 1951. Assay of antibiotics in body fluid by cup method. Rinsho (Clinic) **4**:678.

Morley, D. C. 1945. A simple method of testing the sensitivity of wound bacteria to penicillin and sulfathiazole by the use of impregnated blotting paper discs. J. Pathol. Bacteriol. **57**:379.

Tunneval, G., and H. Ericsson. 1954. Sensitivity test of disc method as a guide for chemotherapy. Antibiotics & Chemotherapy **4**:886.

# ANTIBIOTIC SENSITIVITY TESTING USING PRETREATED AGAR PLATES

## III. Inhibition of *Proteus* Species with a Combination of Sulfathiazole and Colistimethate Sodium

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The search for a drug effective against the various species of the *Proteus* (Hauser) group of gram-negative bacteria has not unearthed many antibiotic or chemotherapeutic substances which are able to demonstrate any marked in vitro inhibition (Kaye, Koenig, and Hook, 1961). This organism has developed a high degree of resistance and adaptability to most antibiotic substances.

The ability of *Proteus* to persist in mixed infections and often to remain the sole survivor after intensive and prolonged antibiotic and chemotherapeutic treatment is also well known to the hospital bacteriologist and clinician (Petersdorf and Hook, 1960).

Since this organism is often recognized by its active motility and swarming on the surface of agar plates, it was hoped that the inhibition of flagellar movement by an effective drug might reduce the uptake of nutrients or alter the surface tension effects on the organism and thereby weaken it sufficiently so that the presence of a second drug could completely inhibit its subsequent growth and division. Previous in vitro studies had indicated that a combination of the drug polymyxin B (a polypeptide) and various sulfonamides at low levels not only inhibited motility but also interfered with subsequent division (Herman, 1959). Unfortunately, pain on injection and other undesirable side effects with polymyxin B made further in vivo studies impractical. However, the introduction by Koyama and associates (1950) in Japan of two new members of the polypeptide family of antibiotics, namely, colistin sulfate and colistimethate sodium (the active principles of Coly-Mycin Pediatric suspension and Coly-Mycin Injectable, Warner-Chilcott Laboratories, Morris Plains, N.J.), provided additional antibiotic compounds known to be effective against most species of gram-negative bacteria except members of the *Proteus* group. The present study was undertaken to determine

the effect on the in vitro growth of *Proteus* cultures with a combination of several soluble sulfonamides and the above polypeptides at varying levels and treatments. The biochemistry and toxicity of these polypeptides have been described elsewhere (Schwartz et al., 1960; Wright and Welch, 1960).

## Materials and Methods

*Media.* Trypticase soy agar (TSA) and broth (Baltimore Biological Laboratory, Baltimore, Md., BBL) were used throughout the experiment except as noted.

*Inoculum.* Kauffmann strain of *Proteus* #94 (F 321), supplied by courtesy of Elizabeth Verder, National Institute of Allergy and Infectious Diseases, National Institutes of Health, was used throughout the experiment. This strain was kept on Trypticase soy agar slants and when required was transferred to a blood agar plate as a single streak and incubated overnight at 37 C to stimulate swarming. A loopful from the edge of the spreading growth was transferred to Trypticase soy broth, incubated for 2 to 3 hr, and 0.5 ml of this culture was added per 300 ml of cooled TSA just prior to pouring the seed layers.

*Test materials.* The colistin sulfate, colistimethate sodium, polymyxin B, and sodium sulfathiazole were each made up aseptically as 0.5% stock solutions with distilled water (5,000 $\mu$g per ml of active base), stored in the refrigerator, and diluted as needed. Commercially available discs (BBL) of colistin sulfate, 10 $\mu$g; polymyxin B, 30 $\mu$g; triple sulfa, 0.25 mg; and sulfamethizole (Thiosulfil, Ayerst Laboratories), 0.25 mg were used as indicated.

TSA was dispensed and sterilized in 300-ml amounts in 500-ml flasks and stock solutions added to provide final concentrations of 2.5, 5.0, and 10.0 $\mu$g of each drug per ml as required; 42 ml of this pretreated cooled agar were poured into each of six 20 by 150 mm plastic plates and allowed to harden. Using a separate plate for each compound to be tested, the appropriate disc was placed on the surface of the pretreated agar about 1 inch from the edge of the plate. The plates were then stored in the refrigerator for 4 to 144 hr to permit diffusion into the agar from the added discs. The agar seed layer was then added (8 ml per 150 mm plastic plate), and an additional disc identical to the one already on the plate was placed 1 inch from the opposite edge of the plate. All plates were then incubated for 24 hr at 37 C, at which time the zone diameters were read in millimeters and recorded.

## Results

The variation in size of the circular zones of inhibition around the discs indicated that the diffusion of the various test substances through the agar base from the discs was continuous during the various holding periods. It may be noted that 2.5 μg of colistimethate sodium per ml of TSA produced a clear zone around the triple sulfa disc (Fig. 1). When diffusion was extended beyond 48 hr, the level

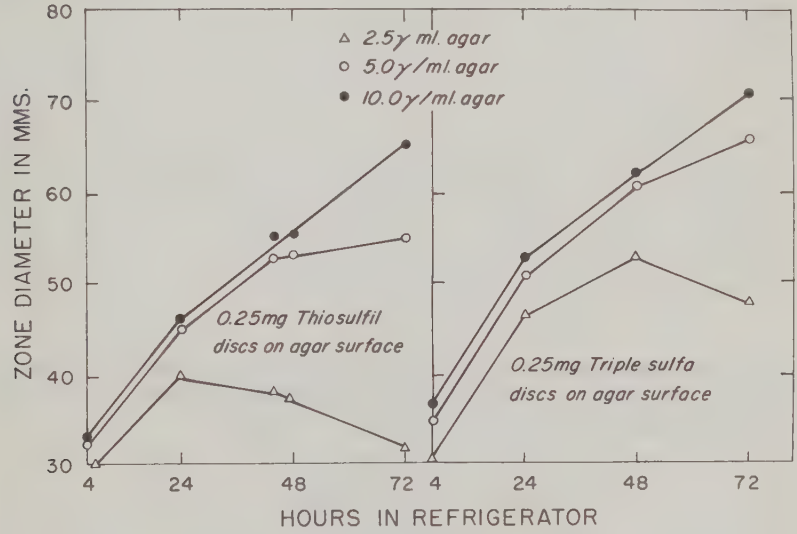


FIG. 1. *Colistinmethanesulfonate sodium in Trypticase soy agar.*

of colistimethate sodium was below that needed to increase the diameter of a clear zone of inhibition around the sulfa disc. On the other hand, there was little difference between 5 and 10 μg/ml of colistimethate sodium even after a 72-hr diffusion period at 4 C. The Thiosulfil disc, slightly less effective than triple sulfa, did not show an increase in size of the inhibition zone at the 2.5-μg level after the 24-hr holding period. A slightly greater difference between the 5 and 10 μg/ml levels of colistimethate sodium in the agar base was also noted.

In Fig. 2 colistin sulfate at the same active base level as colistimethate sodium, i.e., 2.5, 5.0, and 10.0 μg/ml of TSA, showed a slightly higher level of activity with both triple sulfa and Thiosulfil discs, and also indicated no further inhibition in agar with 2.5 μg/ml of TSA after a 48-hr diffusion period. Very little difference was noted between 5- and 10-μg/ml levels. The activity of polymyxin B

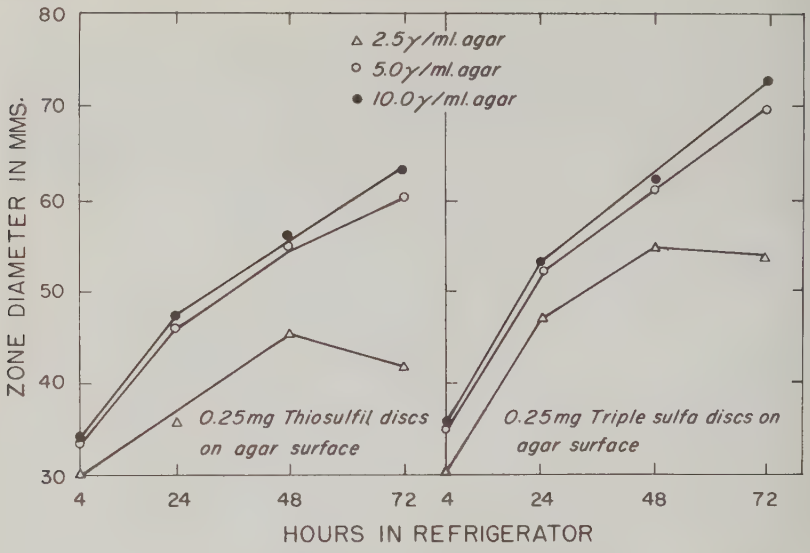


FIG. 2. *Colistin sulfate in Trypticase soy agar.*

resembles that of colistin sulfate except that the zones of inhibition even at 2.5 μg/ml of TSA showed a continuing increase in size up to a 72-hr diffusion period (Fig. 3).

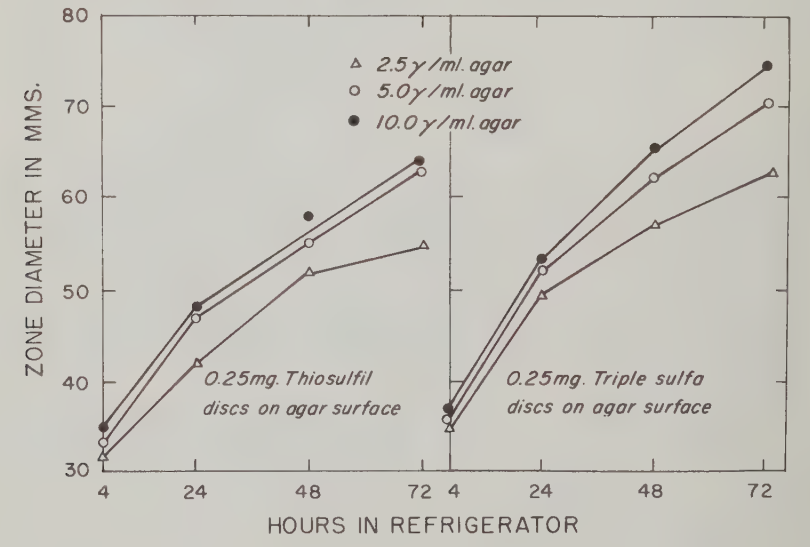


FIG. 3. *Polymyxin B sulfate in Trypticase soy agar.*

By reversing the procedure and placing colistin sulfate and polymyxin B discs on agar containing sulfathiazole (Fig. 4) the zone diameters were much smaller (15 to 30 mm) after the 72-hr diffusion period, which was due both to the slower diffusion rate of the much larger sized molecules of the polypeptides and the lower levels of the drugs in the discs, i.e., 30 $\mu$g of the polymyxin B compared to the 250 $\mu$g of the triple sulfa.

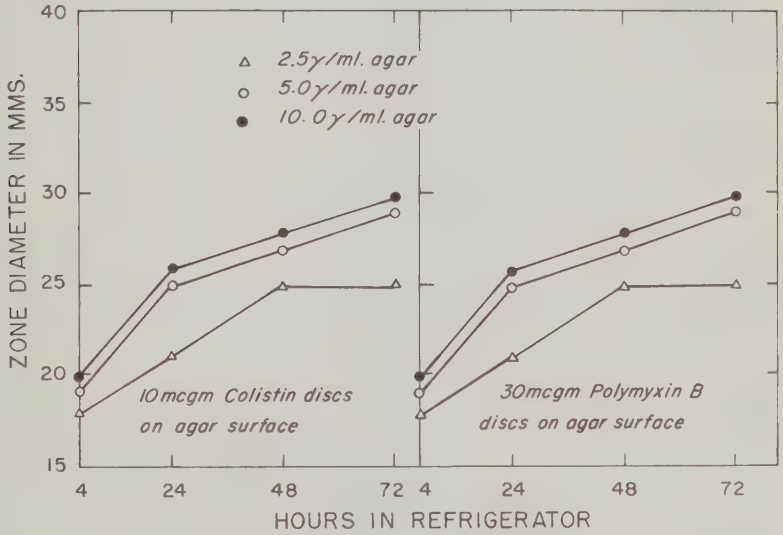


FIG. 4. *Sulfathiazole in Trypticase soy agar.*

The effect on the test of various ingredients contained in the agar base is clearly shown in Fig. 5. Brain heart infusion agar with added *p*-aminobenzoic acid (PABA) completely neutralized the sulfa in the triple sulfa disc and hence no activity was possible. Mueller-Hinton and TSA media, although free from PABA, gave smaller zones of inhibition with the sulfa-colistimethate sodium combination and showed a leveling off of activity after a 96-hr diffusion period. The two other types of media (Fig. 5) showed larger inhibition zones and demonstrated diffusion of the "activating principle" to an amazing 95-mm zone of inhibition after holding the nutrient agar plates for 144 hr. TSA used throughout the experiment, although not the best medium for maximal demonstration of zone diameter, does permit heavier growth for the same level of inoculum and incubation period.

Since the very low level of 2.5 $\mu$g/ml of the sulfathiazole or polypeptide compounds in the media appears to potentiate the second

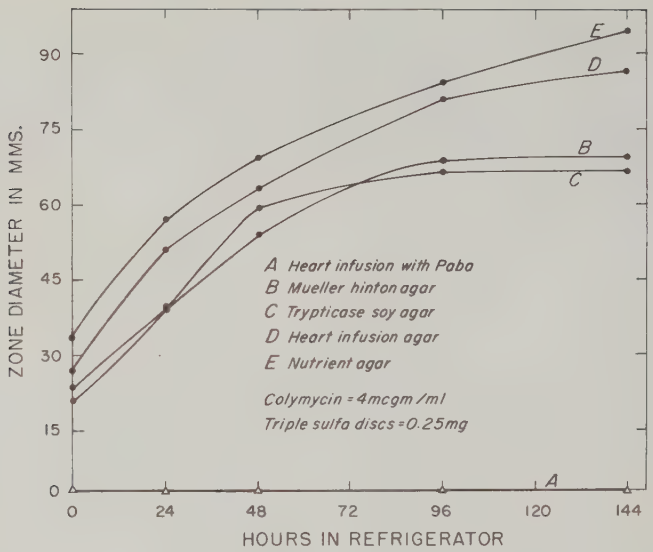


FIG. 5. *Effect of media on zone size.*

compound when the latter is present at high levels in the disc, the minimal effective dose of both compounds was then determined. Table 1 shows the effects of adding equal amounts of sulfathiazole and the polypeptides up to a maximum of 10 μg per ml of TSA base. Colistin sulfate and polymyxin B appear to be slightly more active than the colistimethate sodium at 8 μg per ml; however, no growth was noted when this was raised to the 10 μg per ml level.

TABLE 1. *Effect of combinations of drugs on Proteus in Trypticase soy agar**

| Drug used in combination with sulfathiazole | Reaction to amounts (μg/ml) of each drug in each combination | | | | |
|---|---|---|---|---|---|
| | 1 | 2 | 4 | 8 | 10 |
| Colistimethate sodium | ++++ | ++++ | ++ | 4 colonies | no growth |
| Colistin sulfate | ++++ | ++ | + 12 | no growth | no growth |
| Polymyxin B | ++++ | ++ | + 8 | no growth | no growth |

*Incubation for 24 hr at 37 C.

The results of a test to determine the in vitro inhibition level of each compound alone are noted in Table 2. Sulfathiazole does not

inhibit growth completely even at the 800 μg/ml concentration, and colistimethate sodium permitted growth of a few colonies at this level; however, colistin sulfate and polymyxin B showed almost complete inhibition at 400 μg per ml.

TABLE 2. *Effect of single drugs on Proteus in Trypticase soy agar*

| Drug | Reaction to varying amounts of drug | | | |
|---|---|---|---|---|
| | 100 | 200 | 400 | 800 |
| | μg/ml | μg/ml | μg/ml | μg/ml |
| Sulfathiazole | ++++ | ++++ | ++ nonmotile growth | + nonmotile growth |
| Colistimethate sodium | ++++ | ++++ | ++ | + slow growth |
| Colistin sulfate | +++ | ++ | 10 colonies | no growth |
| Polymyxin B | ++ | 20 colonies | no growth | no growth |

Although the effects of many variables must be taken into account in an experiment of this type, the 40- to 80-fold difference in the amount of each drug necessary to inhibit growth of *Proteus* species when used alone over that when both compounds are present in equal amounts in the media at the same time appears to be an outstanding example of synergism in the presently accepted definition of the word.

## Discussion

Polypeptides occur as large molecules and, therefore, diffuse more slowly through the agar matrix and show a smaller zone of inhibition for the same diffusion period than do the sulfas. For this reason it is much simpler to add the polypeptides to the agar at noninhibitory levels and to permit the sulfa to diffuse from discs, so that any subsequent zones of diffusion and inhibition are clear and distinct. Since polypeptides also show slight surface activity, dead as well as living organisms tend to adsorb them. Thus, when added to agar media at a low level, e.g., 2.5 μg/ml, the polypeptides may eventually disappear due to absorption, and growth from the outer edges of the zone may again spread over the clear area (Fig. 6 and 7).

Amounts of 5 to 10 μg of colistin sulfate per ml of agar appear to be sufficient to potentiate the sulfa diffusing from a 0.25-mg disc to a distance of at least 30 mm on direct incubation and this may extend to 90 mm when the sulfa is permitted to diffuse for periods up to 144 hr at 4 C prior to incubation. Calculating the actual level of the sulfa at the edge of the inhibition zone on the basis of inverse

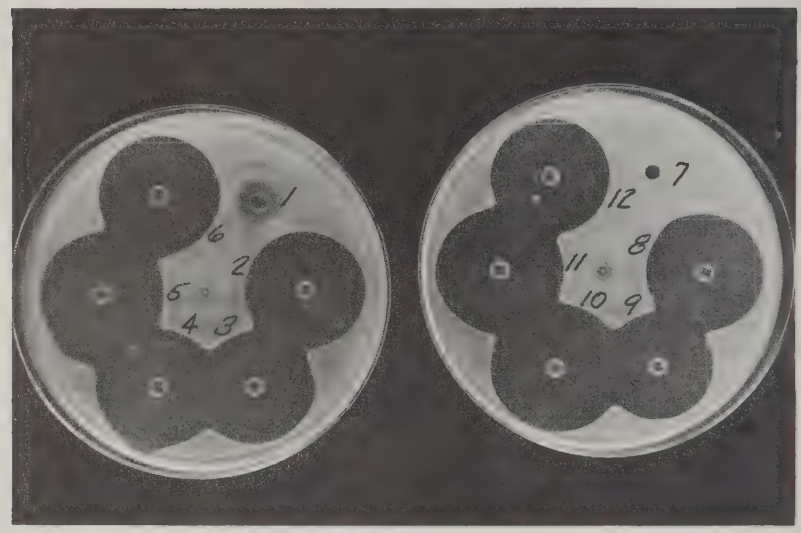


FIG. 6. *Inhibition of Proteus mirabilis by various sulfa compounds, 1 mg per disc, activated by 5 μg of colistimethate sodium per ml of Trypticase soy agar. The discs were allowed to diffuse for 6 hr at 4 C prior to incubation at 37 C. (1) Tetracycline, 30 μg; (2) Sulfadiazine; (3) Sulfamerazine; (4) Thiosulfil; (5) Madribon; (6) Kynex; (7) Polymyxin B, 30 μg; (8) Sul Spansion; (9) Elkosin; (10) Sulfathiazole; (11) Gantrisin; (12) Triple sulfa.*

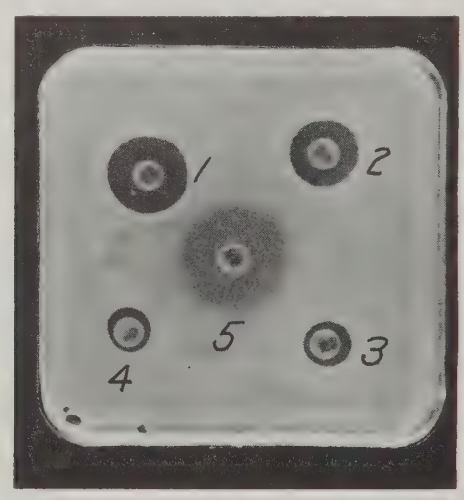


FIG. 7. *Similar to Fig. 6 except that the Trypticase soy agar contains 5 μg sulfathiazole per ml to activate the added polypeptide discs against Proteus mirabilis. (1) Polymyxin B, 30 μg; (2) Colistin sulfate, 10 μg; (3) Polymyxin B, 5 μg; (4) Colistin sulfate, 2 μg; and (5) Thiosulfil, 250 μg.*

proportions, one should find approximately 4 to 10 μg of sulfa present at the edge of the visibly active area. A medium containing PABA neutralizes the effect of sulfathiazole and hence there is no apparent inhibition with added polypeptides. A medium such as TSA or brain heart infusion agar with a high level of nutrients also appears to interfere to some degree. Nutrient agar containing minimal amounts of nutrients therefore shows larger zones of inhibition but slower growth when compared with the enriched media noted above.

The inhibitory potential of each component alone in TSA plates showed that levels 50 to 100 times those known to be effective in combination had little effect on the strain of *Proteus* under test, indicating that there is a distinct potentiation of each of the components by the other. However, the minimal effective in vitro level of equal amounts of each drug is somewhat higher (10 μg/ml) than the activating level (2.5 μg/ml) when either one is in excess of requirements. It should be possible, therefore, to demonstrate an effective in vivo combination for possible patient use where *Proteus* has established itself as a distinct pathogenic process. Serum levels of 8 to 10 μg per ml of each compound are relatively simple to maintain (Pullen, Callahan, and Vial, 1961), especially in the genito-urinary tract where levels of both drugs are, of course, much higher through kidney excretion. Since *Proteus* infections, according to some workers (Kaye, Koenig, and Hook, 1961), are second only to those due to staphylococci, it is hoped that sufficient substantive in vivo information on this point can be obtained in the very near future. The mode of action of these two drugs is not as yet completely understood; however, it is felt that the polypeptide functions as a physical "wedge" (Newton, 1956), making it possible for the sulfathiazole to pass through the cell wall where it appears to interfere with folic acid synthesis of the cell as an analogue of PABA (Elion, Singer, and Hitchings, 1960; Hitchings, 1961).

## Literature Cited

Elion, G. B., S. Singer, and G. H. Hitchings. 1960. Potentiation in combinations of three biochemically related antimetabolites. Antibiotics & Chemotherapy **10**:556-564.

Herman, L. G. 1959. Antibiotic sensitivity testing using pretreated plates. II. A demonstration of inhibitory activity with a low level combination of a sulfonamide and polymyxin B against Proteus species. Antibiotics Ann. 1958-1959, p. 836-839.

Hitchings, G. H. 1961. A biochemical approach to chemotherapy. Trans. N. Y. Acad. Sci. **23**: 700-708.

Kaye, D., M. G. Koenig, and E. W. Hook. 1961. The action of certain antibiotics and antibiotic combinations against Proteus mirabilis with demonstration of in vitro synergism and antagonism. Am. J. Med. Sci. **242**:320-330.

Koyama, Y., A. Kurosawa, A. Tsuchiya, and K. Takakuta. 1950. A new antibiotic "Colistin" produced by spore-forming soil bacteria. J. Antibiotics Japan Ser. A **3**:1950:457-458.

Newton, B. A. 1956. The properties and mode of action of the polymyxins. Bacteriol. Rev. **20**:10-27.

Petersdorf, R. G., and E. W. Hook. 1960. The use of Colistin in the treatment of infections of the urinary tract. Bull. Johns Hopkins Hosp. **107**:133-142.

Pullen, F. W., W. S. Callahan, and A. B. Vial. 1961. A study of bacterial sensitivity to antibiotics: interpretation of "resistance" based upon clinically attainable blood levels. Univ. Mich. Med. Bull. **27**:104-114.

Schwartz, B. S., M. R. Warren, F. A. Barkley, and L. Landis. 1960. Microbiological and pharmacological studies of colistin sulfate and sodium colistinmethanesulfonate. Antibiotics Ann. 1959-1960, p. 41-58.

Wright, W. W., and H. Welch. 1960. Chemical, biological, and clinical observations on colistin. Antibiotics Ann. 1959-1960, p. 61-74.

# USE OF ION-EXCHANGE RESINS IN AGAR FOR ASSAYING ANTIBIOTIC COMBINATIONS IN MILK AND SERUM

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The combining of antibiotics for therapeutic use has created problems when assaying for individual antibiotics. In control laboratories, several methods can be used: differences in solubilities; inactivation of one or more of the antibiotics; use of resistant strains of organisms; classical separation procedures; and use of compensating curves. Most of these methods are inadequate for the assay of antibiotic combinations in milk and serum due to the low level of antibiotics frequently present and the complexity of milk and serum that generally rules out extraction procedures. Also, the Food and Drug Administration requires a very sensitive method for each antibiotic in the combination, so as to detect residual amounts present in milk or blood.

Ion-exchange resins have been used for a number of years in the manufacture, purification, and separation of antibiotics by either the batch or column methods. In agar, their use appears to have been limited to the determination of the ionic character of antibiotics (Sevcik and Podojil, 1957). Brock *(unpublished data)*, working in our laboratories, confirmed their usefulness in this regard.

The purpose of this paper is twofold: first, to describe techniques for binding certain antibiotics, by use of ion-exchange resins, directly in agar, and, second, to give examples, where they were found useful, for detection of antibiotics in milk and serum.

## Materials and Methods

So far, two different resins have been studied. The cationic-exchange resin was Dowex 50 WX4 in the $Na^+$ cycle, 200 to 400 mesh. The resin was supplied in the $H^+$ cycle. For this work it was charged to the $Na^+$ cycle by using concentrated NaOH and washed thoroughly with distilled water until the pH was returned to neutral. While in the $H^+$ cycle, this resin will bind certain antibiotics, but the capacity to bind higher amounts of certain antibiotics was enhanced when in

the $Na^+$ cycle. For the anionic-exchange resin, Dowex 1 X4 in the $Cl^-$ cycle, 200 to 400 mesh, was used and required only washing with distilled water. With both resins, the very fine particle size of 200 to 400 mesh was necessary to obtain binding activity. After washing, the resins were suspended in as little distilled water as possible to obtain a slurry suitable for pipetting. They were then sterilized by autoclaving. The resin in use was added to the seed layer of agar to give concentrations of 1 to 2%.

All assay procedures, for the antibiotics in milk and serum, were generally carried out with a 10-ml base layer and 4-ml seed layer of agar. The standard steel cylinder methods, as outlined by Grove and Randall (1955), and the methods for milk, as outlined by Kirshbaum et al. *(unpublished data)*, were followed. At times, certain modifications were made in the assay procedures to increase the sensitivity for determining low levels of antibiotics, but these changes did not influence the actions of the resins.

The test organisms used to date have included *Bacillus subtilis* UC 564 (spore suspension), *Staphylococcus aureus* 209P, *S. epidermidis* ATCC 12228, *Sarcina lutea* ATCC 9341, and *Escherichia coli* UC 48. The organisms were not affected as to growth or appearance by either resin. The antibiotics tested have included penicillin, neomycin, novobiocin, and dihydrostreptomycin.

For assay tests, cows' milk and serum from several different animal species were used. In some cases antibiotics in combination were added to the milk or serum and in other cases the test materials were obtained from antibiotic-treated animals.

## Results

The effect on zone size obtained with four test antibiotics and with the various test organisms was determined by running standard curves for each in the presence and absence of the resins. The results of these initial studies as carried out in buffer solutions follow.

*Penicillin.* The standard curve obtained for penicillin was not affected by either resin. The zone sizes were similar, regardless of whether Dowex 50 or Dowex 1 was present (Table 1). When resin concentration was increased to 5%, a slight effect on the zone size was observed.

*Neomycin.* The results in Table 2 show that neomycin was not affected by the presence of Dowex 1 but was bound by Dowex 50. Although not shown in the table, the Dowex 50 will bind concentrations as high as 10 $\mu$g/ml. Higher levels have not been tested.

*Novobiocin.* Dowex 50 had no effect on zone sizes obtained with novobiocin while Dowex 1 was effective in binding this antibiotic (Table 3). Much higher levels of novobiocin could be bound by Dowex 1 than shown here.

TABLE 1. *Zone sizes for penicillin in the presence and absence of resins*

| Organism | Penicillin concn | Size | | |
|---|---|---|---|---|
| | | Dowex 1 | Dowex 50 | Plain agar |
| | *units/ml* | *mm* | *mm* | *mm* |
| *S. lutea* | 0.0125 | 11.2 | | 10.0 |
| | 0.025 | 14.1 | | 13.6 |
| | 0.05 | 18.1 | | 17.4 |
| | 0.1 | 21.4 | | 21.2 |
| | 0.2 | 24.9 | | 24.4 |
| *S. aureus* | 0.1 | | 0 | 0 |
| | 0.5 | | 11.0 | 11.3 |
| | 1.0 | | 13.5 | 13.7 |
| | 2.5 | | 16.2 | 16.5 |
| | 5.0 | | 18.5 | 18.3 |

TABLE 2. *Zone sizes for neomycin in the presence and absence of resins*

| Organism | Neomycin concn | Size | | |
|---|---|---|---|---|
| | | Dowex 1 | Dowex 50 | Plain agar |
| | *μg/ml* | *mm* | *mm* | *mm* |
| *S. epidermidis* | 0.25 | 11.6 | 0 | 12.2 |
| | 0.5 | 13.8 | 0 | 14.3 |
| | 1.0 | 16.0 | 0 | 15.8 |
| | 2.0 | 19.4 | 0 | 18.0 |
| | 4.0 | 21.0 | 0 | 19.6 |
| | 8.0 | 23.1 | 0 | 21.4 |
| *B. subtilis* | 0.5 | 11.0 | 0 | 11.1 |
| | 1.0 | 15.7 | 0 | 15.1 |
| | 2.0 | 17.4 | 0 | 17.2 |
| | 4.0 | 19.5 | 0 | 19.9 |
| | 8.0 | 21.6 | 0 | 20.7 |
| *S. aureus* | 0.05 | | 0 | 0 |
| | 0.1 | | 0 | 12.3 |
| | 0.5 | | 0 | 17.2 |
| | 1.0 | | 0 | 19.0 |
| | 2.0 | | 0 | 20.0 |
| | 4.0 | | 0 | 21.4 |

TABLE 3. *Zone sizes for novobiocin in the presence and absence of resins*

| Organism | Novobiocin concn | Size | | |
|---|---|---|---|---|
| | | Dowex 1 | Dowex 50 | Plain agar |
| | *μg/ml* | *mm* | *mm* | *mm* |
| *S. epidermidis* | 0.4 | 0 | 12.5 | 11.6 |
| | 0.5 | 0 | 13.9 | 13.4 |
| | 0.625 | 0 | 15.2 | 14.9 |
| | 0.78 | 0 | 16.9 | 16.0 |
| | 0.975 | 0 | 18.0 | 17.1 |

*Dihydrostreptomycin.* Dihydrostreptomycin behaved like neomycin in not being affected by Dowex 1 but was bound successfully by Dowex 50 (Table 4).

TABLE 4. *Zone sizes for dihydrostreptomycin in the presence and absence of resins*

| Organism | Dihydrostreptomycin concn | Size | | |
|---|---|---|---|---|
| | | Dowex 1 | Dowex 50 | Plain agar |
| | *μg/ml* | *mm* | *mm* | *mm* |
| *B. subtilis* | 0.1 | 0 | 0 | 0 |
| | 0.5 | 12.2 | 0 | 11.0 |
| | 1.0 | 14.2 | 0 | 14.5 |
| | 2.5 | 16.8 | 0 | 16.1 |
| | 5.0 | 17.4 | 0 | 16.8 |

*Polymyxin B.* Preliminary experiments indicate that polymyxin B is not bound by either resin.

Other studies have been done in milk and serum, and results were similar to those obtained with standard buffer solutions. With the organisms tested, the resins had no effect on the rate or type of growth, appearance on plates, or amount of growth. The shape and sharpness of the zones obtained did not appear to be affected either.

The standard curves obtained for the various antibiotics, with and without resins in the agar, were compared, to determine if the resins had any effect on the slopes. The results obtained with neomycin in milk, with and without Dowex 1 in the agar, are shown in Fig. 1. Although the two curves are not perfectly parallel, these results are typical of curves obtained with this antibiotic in milk. Curves for novobiocin with and without Dowex 50 in the agar are presented in Fig. 2. As can be seen, the resin does not affect the slope of the curve. These results further verify the lack of effect on the test organisms by either of the resins.

The use of the resins has been found to have many practical applications and a few of these are given.

*Penicillin and neomycin.* Dogs were injected with a preparation containing penicillin and neomycin, and serum levels of each were determined. To assay for neomycin, penicillinase was added to destroy the penicillin. The penicillin assays were carried out on agar containing Dowex 50 which bound the neomycin and permitted the assay for penicillin, without interference from the neomycin. Similar techniques could be used for a neomycin and novobiocin combination by using the Dowex 1 to bind the novobiocin and Dowex 50 to bind neomycin.

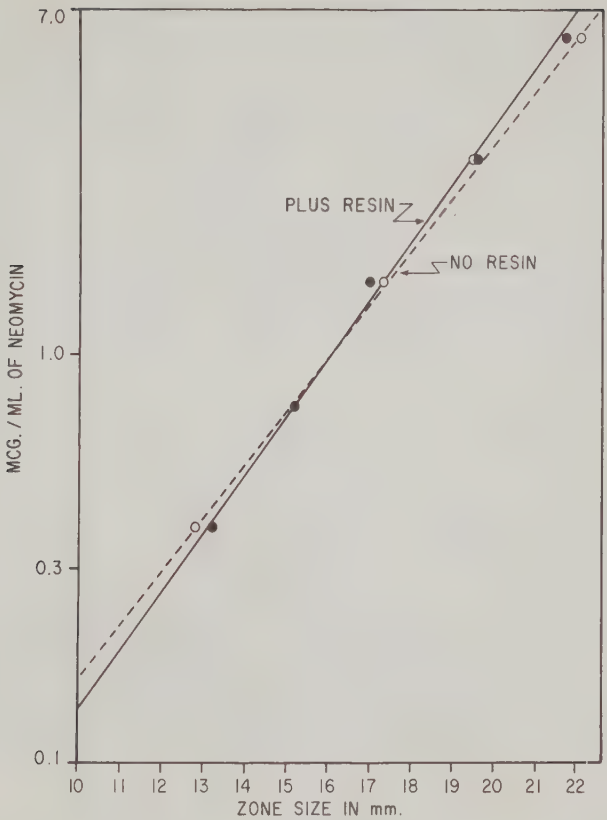


FIG. 1. *Neomycin standard curve with and without Dowex 1 resin.*

*Penicillin and novobiocin.* Milk samples, from cows treated intramammarly with a penicillin and novobiocin preparation, were assayed. The Dowex 1 was used to bind novobiocin when tests were conducted for penicillin.

*Penicillin, neomycin, and polymyxin B.* A preparation containing three antibiotics, penicillin, neomycin, and polymyxin B, was infused into the udder of cows. The neomycin in the milk was assayed by the standard method after the penicillin was destroyed by penicillinase. Since neomycin will interfere with the polymyxin assay, it was bound with Dowex 50, ruling out the necessity of having to run a compensated curve for polymyxin in the presence of neomycin.

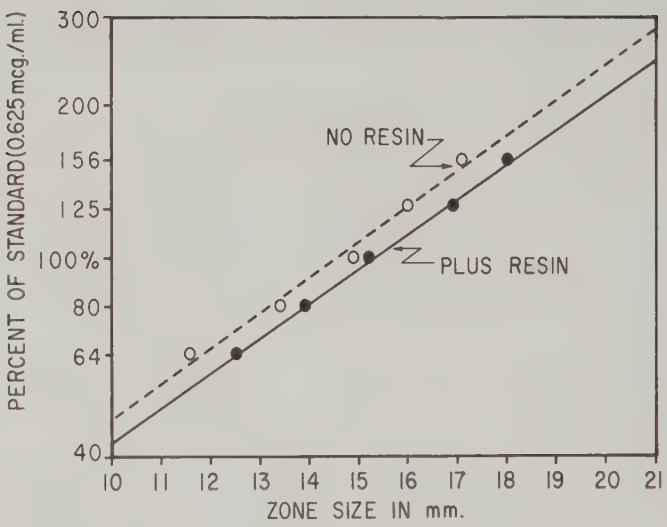


FIG. 2. *Novobiocin standard curve with and without Dowex 50 resin.*

*Four antibiotics.* A preparation containing penicillin, novobiocin, neomycin, and dihydrostreptomycin was used for intramammary infusion into cows. By using agar containing Dowex 50 to bind the neomycin and dihydrostreptomycin, and penicillinase to destroy the penicillin, assays were carried out for novobiocin. Dowex 1 was used to bind the novobiocin while assaying for the other antibiotics.

## Discussion

Although the use of ion-exchange resins in agar to determine the ionic character of antibiotics is not new, the use of these resins in agar as an assay method for antibiotic combinations is new. The use of such resins in microbiology was the subject of a recent review (Rotman, 1960); however, no mention was made of their use for assaying antibiotics.

Thus far, studies have been carried out with Dowex 50 and Dowex 1 resins only. There is a very good possibility that a survey of other resins may enable us to increase the binding spectrum of other antibiotics since the properties and binding capacities of resins are quite varied. The one factor that appears necessary is that extremely fine resins of 200 to 400 mesh are required to obtain the maximum effect. From what is known, the $pK_a$ value of the antibiotic would also be a determining factor in which antibiotics are

bound. In this limited study, only the basic and acidic ones, as a rule, were bound; the amphoteric antibiotics did not appear to be affected. Sevcik and Podojil (1957) reported the binding of streptomycin, neomycin, viomycin, and the partial binding of chlortetracycline and oxytetracycline by the cationic resin. Only penicillin and chloramphenicol, to some degree, were reported by them to be bound by the anionic resin. In studies reported here, penicillin was not bound by either resin.

The use of resins in agar to bind antibiotics has been proven a very valuable assay tool. This was especially true when certain antibiotic combinations had to be assayed in the presence of serum and milk. Without the resins, many of the assays carried out would have been almost impossible. As our information is increased about the uses of resins in agar, it is felt that more and better use can be made of them in assay work.

## Literature Cited

Grove, D. C., and W. A. Randall. 1955. Assay methods of antibiotics, a laboratory manual. Medical Encyclopedia, Inc., New York.

Rotman, B. 1960. Uses of ion exchange resins in microbiology. Bacteriol. Rev. 24:251-260.

Sevcik, V., and M. Podojil. 1957. Orientierende Bestimmung des Ionencharakters der Antibiotika mit Hilfe von Plattentesten. Naturwissenschaften 44:93.

# ACCUMULATION AND DEPOSITION OF TETRACYCLINE ANTIBIOTICS IN BONES OF CHICKENS FED DIETS CONTAINING THESE ANTIBIOTICS

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The supplementation of rations of farm animals with antibiotics, especially with those of the tetracycline series, has in recent years become a common practice. Many times it has been shown that, even when doses of 100 to 200 mg of antibiotic per kg of feed were used, no residues of the antibiotics could be detected in blood serum or meat of the animals. Only after 2 to 3 weeks of feeding antibiotic-supplemented rations, or after doses of 500 to 1000 mg per kg feed, i.e., amounts approaching therapeutic doses, does it become possible to identify small antibiotic residues, usually less than 1 $\mu$g/g. If antibiotics in the diet are discontinued 1 to 2 days prior to slaughtering the animals, even long-term feeding of antibiotics or feeding of high doses fails to leave detectable residues of antibiotics in blood or meat of the animals (Broquist and Kohler, 1954; Freerksen, 1954).

Recently, some authors (Milch, Rall, and Tobie, 1957; Rall et al., 1957; Kelly and Buyske, 1960; Buyske, Eisner, and Kelly, 1960) studied the problem of accumulation of tetracycline antibiotics in bones of experimental animals after therapeutic, especially parenteral, doses of tetracyclines. As a means of detecting the accumulation of tetracyclines in body organs or in bone tissue, these authors used fluorescence of tetracyclines in ultraviolet light. In our country, this problem was studied by Málek and Kolc (1960*a*,*b*,*c*), who improved methods for the study of the distribution of tetracyclines in the organism after ingestion of therapeutic doses, and who proposed a technique of fluorescence photography. They were able to confirm the accumulation and deposition of tetracyclines in some organs during pathological states and the accumulation and deposition of tetracycline antibiotics in bone tissue, especially of young individuals.

In the present work we have studied the distribution of chlortetracycline and especially its deposition in bone tissue of chickens which had been fed 100 mg of the antibiotic per kg of feed. We have also determined the levels of the antibiotic in bone tissue of

these animals. We have found that, with this dose of chlortetracycline, an accumulation in bone tissue of chickens occurs very rapidly, and its concentration exceeds several times the concentrations encountered in meat. It was again verified that, when the addition of the antibiotic to the diet was interrupted for 1 or 2 days, it became impossible to demonstrate its presence either in blood or in meat. At this dosage level (i.e., a small fraction of the therapeutic dose), we observed considerable accumulation and deposition of chlortetracycline in bones of chickens. The amounts present were found to be in the range of 1 to 50 $\mu$g per g of dried bone tissue. The antibiotic persists, deposited in the bones, even when the supplementation of the diet has been interrupted for 1 week.

## Materials and Methods

Chickens (1-week old) were fed a standard diet, supplemented with 100 mg of chlortetracycline per kg of feed. Chickens of group I were fed the antibiotic-supplemented diet for 1 week; after 48 hr, the amount of antibiotic in the bones was determined. Chickens of group II were fed the antibiotic for 1 week and then, after receiving a diet without the antibiotic for another week, the amount of chlortetracycline in the bones was determined. Group III was fed the antibiotic-containing diet for 4 weeks, then 1 week the diet without antibiotic, whereupon the amount of chlortetracycline in bones was determined.

The chickens were killed, scalded, and eviscerated. The bones were removed and the presence of chlortetracycline estimated by fluorescence photography, both macro- and microscopic, and by the quantitative agar diffusion microbiological method.

For the bioassay, bones were dried and comminuted to a fine powder. The powdered bones were extracted by a 2% solution of oxalic acid, the pH of the extract was adjusted to 4.5 by means of phosphate buffer, and the chlortetracycline content of this buffered solution was determined by the standard agar diffusion bioassay (Grove and Randall, 1955), using *Bacillus cereus* var. *mycoides* ATCC 6538 (Hess and Čapková, 1960) as the test organism.

In one series of experiments, the bones were boiled in water for 60 min.

## Results

The surface of the bones was observed to have an intensive yellow-gold fluorescence in ultraviolet light (Philora 57202 E/70), typical for chlortetracycline. In endosteum, strong fluorescence was likewise seen. In animals of groups II and III, where there was an

interval of 1 week between the discontinuation of the antibiotic in the diet and the observation of the fluorescence, the fluorescence was found to be slightly weaker, yet still quite marked (Fig. 1).

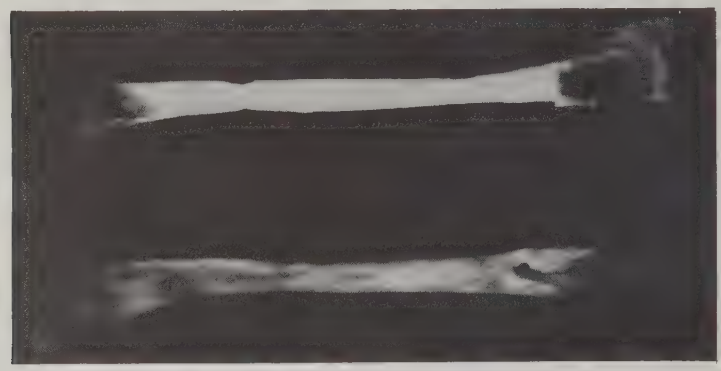

FIG. 1. *(top) Fluorescence of a bone from a chicken fed with chlortetracycline, compared with (bottom) a normal bone.*

The results of bioassay of antibiotics in bone tissue are given in Table 1. A marked accumulation and deposition in bone tissue occurred. Even when the chlortetracycline supplementation was interrupted for 1 week, the antibiotic level in the bone remained almost unchanged. The strikingly high level of chlortetracycline in the bones of chicken no. 1485 is consistent with previous findings (Málek and Kolc, 1960*c*) that in various pathological states therapeutic doses may lead to excessive accumulation of chlortetracycline in the bones. We have now found that this phenomenon occurs even with relatively low feeding doses.

TABLE 1. *Bioassay of chlortetracycline in bones of chickens*

| Group | Chicken no. | Age | Live weight | Chlortetracycline in dried bone | Chlortetracycline in dried bone after 60 min boiling |
|---|---|---|---|---|---|
| | | *weeks* | *g* | *μg* | *μg* |
| I | 1443 | 1 | 57 | 2.50 | 2.32 |
| | 1472 | 1 | 51 | 8.76 | 8.50 |
| | 1448 | 2 | 121 | 2.42 | – |
| | 1467 | 2 | 128 | 7.0 | 7.10 |
| II | 1485 | 2 | 84 | 43.3 | 38.6 |
| | 1497 | 2 | 118 | 3.3 | – |
| III | 1459 | 4 | 270 | 4.48 | – |
| | 1466 | 4 | 260 | 5.72 | 5.42 |

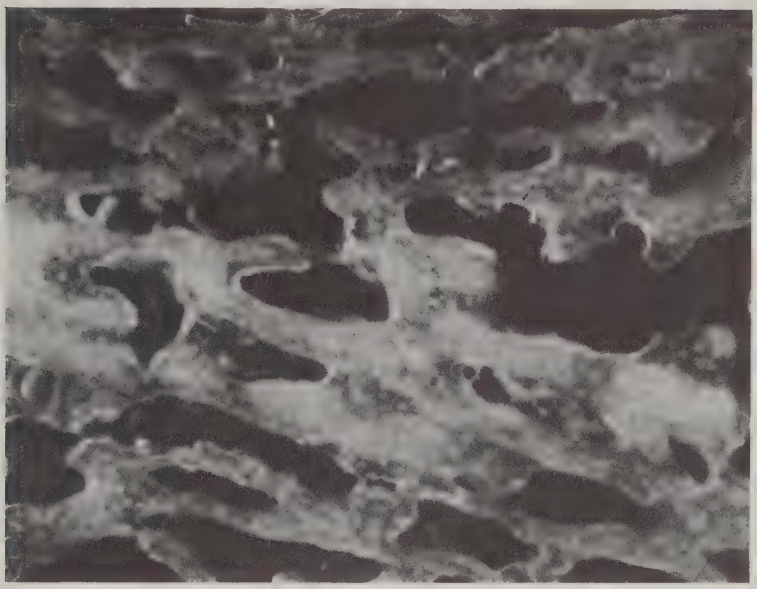

FIG. 2. *Photomicrograph of bone tissue from a chicken fed with chlortetracycline.*

Boiling of bones for 60 min failed to decrease the fluorescence intensity, which means that the chlortetracycline-bone complex is heat-stable.

The finding that tetracycline antibiotics (chlortetracycline) are persistently accumulated and fixed in bone tissue of animals fed relatively low doses is considered important, although no accumulated residues of the antibiotics could be found either in the blood serum or in the meat of animals. Fortunately, the fact that chlortetracycline remains deposited in the bones, even upon boiling in water, leads us to presume that it does not pass into food during the cooking of antibiotic-fed poultry.

## Literature Cited

Broquist, H. P., and A. R. Kohler. 1954. Studies of the antibiotic potency in the meat of animals fed chlortetracycline. Antibiotics Ann. 1953-1954, p. 409-415.

Buyske, D. A., H. J. Eisner, and R. G. Kelly. 1960. Concentration and persistence of tetracycline and chlortetracycline in bone. J. Pharmacol. Exptl. Therap. **130**:150-156.

Freeksen, E. 1954. Lecture held at the Symposium on antibiotics in animal nutrition. Munich, Lederle Mitteilungen, p. 5-6.

Grove, D. C., and W. A. Randall. 1955. Assay methods of antibiotics. Medical Encyclopedia, Inc., New York.

Hess, J., and J. Čapková. 1960. Determination of chlortetracycline in animal tissues. Primysl potravin **11**:331-333.

Kelly, R. G., and D. A. Buyske. 1960. Metabolism of tetracycline in the rat and the dog. J. Pharmacol. Exptl. Therap. **130**:144-149.

Málek, P., and J. Kolc. 1960*a*. Fluorescence method for the determination of the penetration of chlortetracycline into tissues. Časopis lékáu českých. **99**:924-927.

Málek, P., and J. Kolc. 1960*b*. The effect of fractures on the distribution of tetracycline antibiotics in bones. Acta chirurg. orthoped. traumatol. **27**:396-404.

Málek, P., and J. Kolc. 1960*c*. Penetration of chlortetracycline into tissue affected by pathological changes. Antibiotics & Chemotherapy **10**:488-494.

Milch, R. A., D. P. Rall, and J. E. Tobie. 1957. Bone localization of the tetracyclines. J. Natl. Cancer Inst. **19**:87-91.

Rall, D. P., T. L. Loo, M. Lane, and M. G. Kelly. 1957. Appearance and persistence of fluorescent material in tumor tissue after tetracycline administration. **17**:79-84.

# IN VITRO STUDIES OF ACTINOSPECTACIN

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Actinospectacin (Trobicin) was recently described as a new broad-spectrum antibiotic (Mason, Smith, and Dietz, 1961; Hanka, Mason, and Sokolski, 1961; Lewis and Clapp, 1961; Bergy, Eble, and Herr, 1961). The in vivo response of actinospectacin in experimental infections was greater than might be expected from its in vitro activity in brain heart broth (Lewis and Clapp, 1961). Sokolski, Chidester, and Schadewald (1961) found the in vitro activity of actinospectacin in human whole blood comparable to that in modified brain heart broth and more correlative with in vivo activity. In two media, human whole blood and modified brain heart broth, actinospectacin in vitro activity approached that of chloramphenicol.

The effects of media, pH, and reversing agents are reported in the present paper. Their significance in the problem of sensitivity testing on media routinely used in clinical laboratories is discussed.

## Materials and Methods

The following media were used in various phases of this study: SAA, streptomycin assay agar with yeast extract (Baltimore Biological Laboratory), pH 7.9; TSA, Trypticase soy agar (Baltimore Biological Laboratory), pH 7.1; BBA, blood base agar + 5% whole human blood (Difco), pH 6.7; BHIA, brain heart infusion agar (Difco), pH 7.3; BHIB, brain heart infusion broth (Difco), pH 7.3; and SAB, streptomycin assay broth with yeast extract (Difco), pH 7.6.

SAB has the following composition: beef extract (Difco), 1.5 g per liter; yeast extract (Difco), 3.0 g per liter; peptone (Difco), 6.0 g per liter; the presterilization pH was adjusted to 7.9.

In those investigations concerned with variations in the pH of the media, HCl or NaOH was used to adjust the pH of the media after sterilization.

Actinospectacin sulfate (662 $\mu$g per mg, base equivalents) was used for all of the experiments, and the results are expressed in $\mu$g of base equivalents.

Twofold dilution end points were run in 18 × 50 mm tubes, 5 ml per tube. The tubes were inoculated with approximately $10^6$ organisms per ml of the respective bacteria being studied. Actinospectacin

was dissolved in water and diluted into the various media being studied. Chloramphenicol was dissolved in a minimal amount of ethanol and subsequently diluted into the various media. Minimal inhibitory concentrations (MIC) are reported as $\mu$g per ml to completely inhibit growth after 18 hr of stationary incubation at 37 C.

Agar diffusion disc plate experiments were conducted with 10 ml of medium per petri dish (20 × 100 mm). The media were seeded at the rate of about $10^6$ organisms per ml of agar. Actinospectacin sensitivity discs (6.35 mm) were prepared with 5, 30, 60, 100, 300, 600, and 1,000 $\mu$g per disc. The antibiotic was dissolved in deionized water; the discs were dried prior to use. Sensitivity discs (Baltimore Biological Laboratory) of other antibiotics were run in parallel.

Resistance development to actinospectacin was studied by making serial transfers of *Staphylococcus aureus* UC-80 in actinospectacin-containing broths. Twofold dilution end points were run every 24 hr at 37 C in SAB (pH 6.5, pH 7.5) and BHIB (pH 7.3); the inoculum for each day was from the previous day's tube which showed growth in the highest concentration of drug. Sensitivity testing was performed as described above.

Various probable media components were studied for in vitro antagonism or synergism manifestations. These agents were incorporated (0.01 and 0.1%, w/v) in agar and broth used for assaying actinospectacin. Any agents showing significant deviations from the controls received more extensive study.

Unless otherwise specified, all inocula were prepared from overnight cultures in BHIB. The bacteria were incubated without agitation at 37 C, with the exception of *Pseudomonas fluorescens* which was incubated at 28 C.

## Results

The preliminary results of this study confirmed the previously described observations (Lewis and Clapp, 1961) that actinospectacin exhibits a low order of in vitro activity in brain heart infusion and similar "rich" media. Our present studies show that actinospectacin is more active in streptomycin assay medium with yeast extract at pH 7.6 than in brain heart infusion media. Table 1 lists the broth dilution end points of actinospectacin in SAB and BHIB. Chloramphenicol end points, determined under identical conditions, are included for comparison. These results with 22 different organisms representing 33 strains show that the MIC of actinospectacin in SAB are 5 to 10 times lower than those obtained in BHIB and comparable to those obtained with chloramphenicol.

The pH of the assay medium has a considerable effect on the assay of basic antibiotics, including actinospectacin. Table 2 lists

TABLE 1. *Minimal inhibitory concentration of actinospectacin and chloramphenicol in brain heart infusion broth and streptomycin assay broth with yeast extract*

| Organism | Strain no. | MIC (μg/ml) | | | |
|---|---|---|---|---|---|
| | | Actinospectacin | | Chloramphenicol | |
| | | SAB | BHIB | SAB | BHIB |
| *Staphylococcus aureus* | 76 | 16.5 | 83 | 12.5 | 3.0 |
| | 391 | 66 | 83 | 12.5 | 3.0 |
| | 488 | 16.5 | 83 | 12.5 | 100 |
| *Diplococcus pneumoniae* | 41* | 4.1 | 21 | 3.1 | - |
| | 42* | 4.1 | 21 | 3.1 | 0.78 |
| *Streptococcus hemolyticus* | 754 | 2.1 | 21 | 3.1 | 0.78 |
| | 755 | 2.1 | 42 | 0.78 | 0.39 |
| | 759 | 8.3 | 21 | 6.2 | 1.5 |
| *Streptococcus faecalis* | 157 | 16.5 | 165 | 12.5 | 6.0 |
| | 700 | 16.5 | 83 | 6.2 | 12 |
| *Clostridium perfringens* | 247 | 66 | 165 | 3.1 | 12 |
| *Aerobacter aerogenes* | 441 | 4.1 | 41 | 3.1 | - |
| *Escherichia coli* | 527 | 4.1 | 41 | 6.2 | 3.0 |
| | 557 | 66 | 330 | 6.2 | - |
| *Proteus morganii* | 340 | 8.3 | 41 | 6.2 | 12 |
| | 346 | 0.52 | 83 | 6.2 | >100 |
| *Proteus rettgeri* | 339 | 4.2 | 10.2 | 6.2 | 6.0 |
| | 342 | 8.3 | 83 | 12.5 | 12.5 |
| *Proteus vulgaris* | 232 | 8.3 | 83 | 6.2 | 25 |
| *Pseudomonas aeruginosa* | 348 | >100 | 165 | >100 | - |
| | 350 | 33 | 83 | >100 | 100 |
| *Pseudomonas fluorescens* | 3060 | >100 | -- | >100 | - |
| *Salmonella enteritidis* | 114 | 4.1 | 41 | 6.2 | 1.5 |
| *Salmonella gallinarum* | 265 | 4.1 | 83 | 12.5 | - |
| *Salmonella paratyphi* A | 260 | 4.1 | 21 | 3.1 | 0.78 |
| *Salmonella paratyphi* B | 262 | 16.5 | 83 | 3.1 | 3.1 |
| *Salmonella typhimurium* | 268 | 8.3 | 330 | 3.1 | - |
| *Salmonella typhosa* | 215 | 4.1 | 21 | 3.1 | - |
| *Shigella dysenteriae* | 135 | 8.3 | 83 | 0.78 | - |
| *Shigella paradysenteriae* | 134 | 4.1 | 21 | 6.2 | 0.78 |
| *Shigella flexneri* | 144 | 8.3 | 41 | 12.5 | - |
| *Klebsiella pneumoniae* | 58 | 4.1 | 21 | 1.5 | - |
| | 272 | 4.1 | 21 | 100 | 50 |

*Inoculum increased 10-fold.

the broth dilution end points for *Escherichia coli*, *Proteus vulgaris*, and *S. aureus* in BHIB and SAB at three pH levels. In all instances, actinospectacin is more active in media at pH 8.0 than in those at pH 6.5 and 7.3. Chloramphenicol, as an example of a neutral compound, is not pH-sensitive. Disc-sensitivity testing of actinospectacin is similarly dependent upon pH of the medium. Table 3 lists the zones of inhibition obtained on *Klebsiella pneumoniae* plates at pH 6.5, 7.3, and 8.0. The zones for actinospectacin are largest at pH 8.0 on SAA and BHIA. Similar results were obtained with *S. aureus* UC-76, *E. coli* UC-51, and *P. vulgaris* UC-93. Chloramphenicol was

TABLE 2. *Minimal inhibitory concentrations of actinospectacin and chloramphenicol as influenced by the pH of the medium*

| Antibiotic | Medium | pH | *E. coli* UC-51 | *P. vulgaris* UC-93 | *S. aureus* UC-76 |
|---|---|---|---|---|---|
| | | | *μg/ml* | *μg/ml* | *μg/ml* |
| Actinospectacin | BHIB | 6.5 | 83 | >166 | 83 |
| | | 7.3 | 21 | 83 | 21 |
| | | 8.0 | 5.2 | 41 | 10 |
| | SAB | 6.5 | 10 | 41 | 5.2 |
| | | 7.3 | 5.2 | 21 | 1.3 |
| | | 8.0 | 1.3 | 5.2 | 0.33 |
| Chloramphenicol | BHIB | 7.3 | 4 | 2 | 2 |
| | | 8.0 | 4 | 2 | 4 |
| | SAB | 6.5 | 2 | 0.5 | 8 |
| | | 7.3 | 4 | 1 | 4 |
| | | 8.0 | 4 | 1 | 4 |

only slightly more active at pH 8.0 than at pH 6.5 and 7.3. The sensitivity testing of 30 clinical isolates confirmed the above studies which had been carried out on laboratory-maintained cultures.

TABLE 3. *Agar-plate disc diffusion assay of actinospectacin and chloramphenicol on SAA and BHIA at pH 6.5, 7.3, and 8.0 against K. pneumoniae UC-57*

| Medium | pH of agar | Zones of inhibition | |
|---|---|---|---|
| | | Actinospectacin 30 μg/disc | Chloramphenicol 30 μg/disc |
| | | *mm* | *mm* |
| SAA | 6.5 | 20 | 35 |
| | 7.3 | 25 | 34 |
| | 8.0 | 28 | 36 |
| BHIA | 6.5 | 15 | 28 |
| | 7.3 | 19 | 29 |
| | 8.0 | 22 | 30 |

For the sensitivity testing of isolates against actinospectacin on BHIA and BBA, at the pH commonly used, the activity of discs with up to 1,000 μg per disc was compared on BHIA, BBA, and SAA. Since it appeared that the in vivo potential of actinospectacin is estimated by its in vitro activity on SAA, the inhibition obtained with a 30-μg actinospectacin disc on SAA (pH 8.0) was chosen as the standard. Table 4 shows that 300-μg discs of actinospectacin on BHIA and BBA show sensitivities similar to those of 30-μg discs on SAA. In further evaluations against 23 gram-negative clinical isolates, the 300-μg disc on BHIA, TSA, and BBA gave comparable results with the 30-μg disc on SAA. Against 17 staphylococci the 300-μg disc

on BHIA, TSA, and BBA gave zones slightly larger than those obtained from the 30-μg disc on SAA.

TABLE 4. *Effect of disc concentration on the inhibition zones of actinospectacin on SAA, BHIA, and BBA.*

| Organism | Medium | Actinospectacin (μg/disc) | | | | | | | Chloramphenicol (μg/disc) | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | 1000 | 600 | 300 | 100 | 60 | 30 | 5 | 30 | 5 |
| *S. aureus* UC-76 | BHIA | 24 | 21 | 19 | 17 | 14 | 11 | tr[a] | 25 | 19 |
| | BBA | 22 | 20 | 19 | 16 | 14 | 12 | 10 | 20 | 15 |
| | SAA | | | | | | 20 | 14 | 22 | 16 |
| *P. vulgaris* UC-93 | BHIA | 24 | 22 | 21 | 18 | 14 | 12 | tr | 28 | 20 |
| | BBA | 21 | 20 | 18 | 14 | - | - | - | 25 | 21 |
| | SAA | | | | | | 18 | 12 | 33 | 26 |
| *E. coli* UC-51 | BHIA | 25 | 24 | 23 | 20 | 18 | 18 | 11 | 23 | 16 |
| | BBA | 25 | 23 | 21 | 19 | 17 | 16 | 9 | 19 | 14 |
| | SAA | | | | | | 24 | 19 | 25 | 20 |
| *K. pneumoniae* UC-57 | BHIA | 26 | 25 | 23 | 21 | 19 | 18 | 12 | 26 | 22 |
| | BBA | 26 | 25 | 23 | 20 | 19 | 17 | 11 | 26 | 19 |
| | SAA | | | | | | 23 | 19 | 29 | 25 |

The rate of in vitro resistance development of *S. aureus* UC-80 to actinospectacin is illustrated in Fig. 1. Resistance development

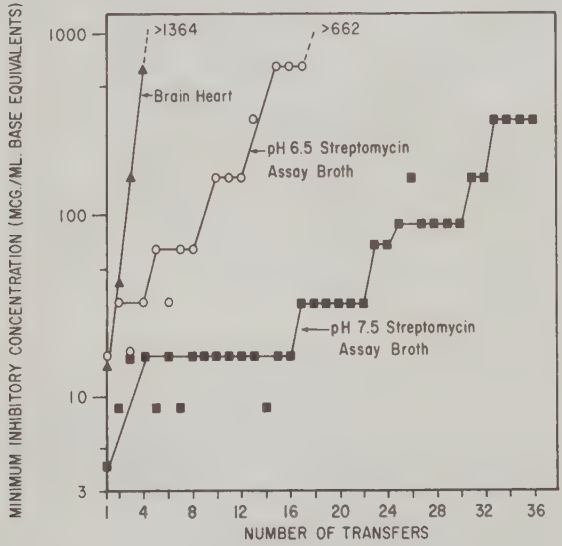


FIG. 1. *Resistance development of S. aureus UC-80 to actinospectacin in brain heart broth, streptomycin assay broth (pH 7.5), and streptomycin assay broth (pH 6.5).*

is less rapid in SAB than in BHIB, and it is significantly diminished at high pH. Cultures resistant to actinospectacin were all found sensitive to the commonly used antibiotics.

A large number of amino acids, vitamins, nucleosides, nucleotides, minerals, and miscellaneous chemicals were screened for reversing or enhancing activity with actinospectacin. At the present time, only one reversing activity has been found. Cysteine, glutathione, and other compounds with reduced sulfur groups showed reversing activity at concentrations of 0.001 to 0.01 M in both the agar-diffusion and the broth-dilution tests. Methionine, cystathionine, cystine, and S-methyl cysteine did not reverse the activity of actinospectacin.

## Discussion

The clinical selection of an antibiotic is accomplished by the demonstration of in vitro sensitivity of the infecting organism(s). Most antibiotics are conventionally evaluated with 5- or 30-$\mu$g discs on brain heart, Trypticase soy, blood base, or similar agars. Several antimicrobial agents, especially the sulfonamides, require much higher concentrations per disc to permit a proper in vitro evaluation of the drug's potential usefulness in the clinic.

Should clinical evaluation of actinospectacin confirm the in vivo activity reported by Lewis and Clapp (1961), the most useful diagnostic test would seem to be either a 30-$\mu$g sensitivity disc applied to SAA, or a 300-$\mu$g disc applied to BHIA, TSA, or BBA with 5% blood.

Actinospectacin apparently does not interfere in cysteine metabolism, since none of the cysteine analogues tested showed the reversing activity of cysteine.

Future studies will be aimed at elucidating the mechanism by which resistance to actinospectacin develops at a different rate in different media. These will require analysis of the role of reversing agents and the complex effects of pH on total cell growth and cell permeability.

## Literature Cited

Bergy, M. E., T. E. Eble, and R. R. Herr. 1961. Actinspectacin, a new antibiotic. IV. Isolation, purification and chemical properties. Antibiotics & Chemotherapy 11:661-664.

Hanka, L. J., D. J. Mason, and W. T. Sokolski. 1961. Actinospectacin, a new antibiotic. II. Microbiological assay. Antibiotics & Chemotherapy 11:123-126.

Lewis, C., and H. W. Clapp. 1961. Actinospectacin, a new antibiotic. III. *In vitro* and *in vivo* evaluation. Antibiotics & Chemotherapy 11:127-133.

Mason, D. J., R. M. Smith, and A. Dietz. 1961. Actinospectacin, a new antibiotic. I. Discovery and biological properties. Antibiotics & Chemotherapy 11:118-122.

Sokolski, W. T., C. G. Chidester, and L. K. Schadewald. 1961. Human whole blood as a medium for testing inhibition by antibacterial substances. Bacterial Proc., p. 63.

# USE OF A REPLICA PLATING TECHNIQUE TO EVALUATE CHANGES IN SKIN FLORA FOLLOWING TOPICAL USE OF HEXACHLOROPHENE OR A NEW IODINE FORMULATION

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Recently, a complex of iodine has been prepared for use as a general skin cleansing and antimicrobial agent. This formulation, called Ioprep (Johnson & Johnson, New Brunswick, N.J.), consists of a 5% concentration of a complex compound of iodine and a nonionic surface-active agent, nonylphenoxypolyoxyethylene ethanol, dissolved in water. The preparation contains 1% available iodine. It also contains a 5% concentration of another similar nonionic surface-active agent which stabilizes the formulation and retains the iodine complex in solution. This iodine compound possesses the antimicrobial activity of iodine but lacks the undesirable properties associated with the topical use of tinctures and solutions of unbound iodine.

Since hexachlorophene is used routinely at this and many other hospitals as a presurgical skin antiseptic, it became of interest to compare its antimicrobial action with that of the iodine complex in a number of human subjects, using the replica plating technique described by Verdon (1961).

## Materials and Methods

For culturing purposes, one inch-square blocks with velvet backing were employed. In obtaining cultures, a sterilized block was first pressed against a blood agar plate, firmly applied to the subject's skin, and again pressed against the same area of the plate. This allowed four blocks to be used per culture plate. In this manner, five areas on one side of each of 10 human subjects were cultured, before and after application of hexachlorophene, and another five areas on the other side of the same individuals, before and after application of the iodine compound. Comparable body sites on each side of the individual were cultured throughout for both preparations, the only difference in the entire procedure being in the scrub

time. After control cultures were obtained, each of the five hexachlorophene sites on each subject were scrubbed for a 10 min-period, whereas the iodine sites were scrubbed for 3 min each. In all cases, a 30 min-period was allowed to elapse, after completion of the scrub, before cultures were again obtained from the same skin site as controls. The scrub times were different for the two preparations because hexachlorophene is used routinely at this hospital for a 10-min period, whereas a 3-min scrub is recommended for the iodine preparation.

The medium used throughout was blood agar which contained 1% bovine albumin, as a neutralizer for hexachlorophene carry-over, or 1% sodium thiosulfate, as a neutralizer for iodine carry-over. These chemicals were present in all plates, including controls.

All plates were incubated at 37 C for 48 hr and the number and types of colonies enumerated.

## RESULTS

The results shown in Table 1 are the composite colony counts of the five areas of each subject before and after application of the antiseptic.

TABLE 1. *Diminution of skin bacteria following application of hexachlorophene or iodine*

| Subject number | Hexachlorophene | | Iodine | |
|---|---|---|---|---|
| | Total no. of colonies of 5 sites prior to use of chemical | Total no. of colonies of sites after 10-min scrub and 30-min interval | Total no. of colonies of 5 sites prior to use of chemical | Total no. of colonies of sites after 3-min scrub and 30-min interval |
| 1 | 295 | 24 | 240 | 1 |
| 2 | 330 | 1 | 262 | 2 |
| 3 | 277 | 0 | 287 | 3 |
| 4 | 67 | 1 | 43 | 1 |
| 5 | 237 | 3 | 145 | 0 |
| 6 | 130 | 0 | 134 | 0 |
| 7 | 113 | 2 | 45 | 3 |
| 8 | 63 | 1 | 335 | 1 |
| 9 | 13 | 2 | 16 | 0 |
| 10 | 73 | 2 | 33 | 1 |
| Totals | 1,598 | 36 (97.7% reduction) | 1,540 | 12 (99.2% reduction) |

It is apparent that both hexachlorophene and iodine greatly reduce the numbers of skin bacteria after contact with the skin for 40 and 33 min, respectively. There is little, if any, difference in the activity of the two preparations as revealed by the present technique.

As would be expected, there was a wide variation in control counts among the 10 subjects.

The organisms encountered in these cultures were those of the normal skin flora, namely, staphylococci, diphtheroids, nonpathogenic *Neisseria,* and *Bacillus* sp. The survivors, for the most part, after application of either antiseptic were *Staphylococcus albus* and gram-positive bacilli of the spore-forming variety.

## Discussion

Use of sterilized one inch-square blocks with velvet backing has made it possible to more nearly standardize tests designed to determine or compare the effect of topical agents on the skin flora. Exact skin areas can be cultured in this manner, before and after the application of chemicals, a condition which is difficult, if not impossible, to achieve with cotton swabs. With this replica method, the bacteria from a definite skin area can be transposed to the surface of an agar plate and the process duplicated repeatedly.

Both hexachlorophene and the iodine complex studied exhibited a marked effect on the microbial flora of the skin. One would conclude that either agent has considerable value as a presurgical skin antiseptic. The only advantage of the iodine compound over hexachlorophene might be the reduced scrub time needed when using iodine.

Previous work by us, using conventional cotton swabs instead of replica blocks, also showed the excellent and similar antimicrobial activities of these two skin antiseptics applied locally.

## Literature Cited

Verdon, P. E. 1961. Efficiency tests on a series of common skin antiseptics under ward conditions. J. Clin. Pathol. 14:91-93.

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